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Acta pharmacol et toxicol 1963, 20, 1–15

From the Department of Pharmacology, University of Goteborg, Sweden
(Professor A Carlsson, M D)

Comparative Effects of Intra-arterially Administered Hydralazine and Sodium Nitrite on Blood Flow and Volume of Forearm[1])

By

B Åblad and G. Johnsson

(Received October 30 1962)

The hypotensive effect of hydralazine is due to a decrease in peripheral vascular resistance (MOYER *et al* 1951, WILKINSON *et al* 1952; FREIS *et al* 1953) In man the point of action seems to be predominantly in the peripheral vascular bed (STUNKARD *et al* 1954, ÅBLAD 1959, ÅBLAD *et al* 1961), where the drug produces a vasodilatation by a "direct" action on the vascular smooth muscle (ÅBLAD *et al* 1962) The effects of systemically administered hydralazine on the resistance in various "parallel-coupled" vascular regions in man seem to be consistent with such a peripheral action Thus the drug induces a distinct increase of blood flow in organs such as the kidneys, the brain and the heart, where the sympathetic vasoconstrictor fibre system is considered to be of little importance for maintaining the vascular resistance under basal conditions (for references see ÅBLAD *et al* 1962)

The effects of systemically administered hydralazine on the vascular resistance in various organs have been studied extensively and are well established However, a vascular bed has a much more complex function than simply to control the resistance to blood flow In fact, each circuit can be divided into several functionally differentiated "series-coupled" sections like Windkessel, resistance, sphincter, shunt, capacitance and exchange vessels" (for definitions see FOLKOW 1959, MELLANDER 1960) Though much work thus has been done to elucidate the effect of hydralazine on the resistance vessels, there seems to have been no investigation directly concerned with the effects on the other "series coupled" sections in the peripheral vascular bed Information on this matter is required for

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any satisfactory description of the mechanisms involved in the general haemodynamic action of hydralazine

In our study we attempted to investigate the effects of hydralazine not only on the resistance vessels but also on the vessels that control the peripheral vascular capacity of blood, i e predominantly the venules and veins (below called "capacitance vessels" as proposed by MELLANDER 1960) Hydralazine was administered at a low dose into a brachial artery, and a comparison was made of the effects on the resistance and capacitance vessels *in the upper part of the forearm* For comparison, a similar investigation was made with sodium nitrite, another drug with a "direct" vasodilator action on vascular smooth muscle The experimental conditions made it possible to study the effects on the resistance vessels by recording the changes of blood flow in the upper part of the forearm The effects on the capacitance vessels were deduced from changes in the continuously recorded forearm volume

Methods

The experiments were performed on healthy recumbent students at a room temperature of 23 ± 1°C For infusion of the drugs a thin polyethylene catheter connected to a motor-driven syringe was introduced into a brachial artery at elbow level, as previously described (ÅBLAD *et al* 1961) Plethysmographs were applied to the upper part of both forearms, and they were filled with water maintained at a constant temperature of 34°C

The measurement of the *blood flow in the forearms* was made by venous occlusion plethysmography, as previously described (ÅBLAD *et al* 1961) Flow was determined simultaneously in both forearms about once a minute Continous measurements of the *changes in the volume of the two forearms* were made separately from the records of blood flow During this procedure the arms were kept in the same position as during the periods of recording blood flow The upper part of the forearms lay at the level of the sternal angle, and the water level in the vertical tube of the plethysmographs was about 10 cm higher than the upper surface of the forearms During the volume recordings the subjects were instructed to lie absolutely still and relaxed It was considered desireable to maintain isovolumetric conditions in the forearms before the drug was given We found that this could be accomplished by maintaining a constant pressure of 20 cm water in the proximal occlusion cuffs during the volume measurements Hand circulation was occluded during the period of forearm volume recording and throughout all drug infusions by maintaining a suprasystolic pressure in the distal cuffs

In one series of experiments simultaneous measurements were made of the changes of volume and *venous pressure in the forearms* before, during and after administering hydralazine or sodium nitrite In a subcutaneous vein near the elbow on the volar part of each forearm a "Rochester" plastic needle was introduced (KNUTH *et al* 1958) This was connected to an open vertical glass tube outside the plethysmograph and the venous pressure was measured by subtracting the height of the fluid level in the vertical tube of the plethysmograph from the height of the level of the fluid column

in the glass tube Each catheter used for recording the venous pressure was connected to a motordriven syringe which infused isotonic saline containing 0 005% heparin at a constant rate of 0 3 ml per minute

The drugs used were hydralazine (1 hydrazinophtalazine apresoline ® Ciba) and sodium nitrite A continuous infusion of drugs or of isotonic saline was given through the intra arterial catheter at a constant rate between 0 3 and 0 6 ml per minute in different experiments Hydralazine was infused for five minutes at a rate of 0 024–0 071 mg per minute Sodium nitrite was in most experiments infused for about 16 minutes at a rate of 0 36–0 89 mg per minute

Statistical analysis was based on the *t* test (FISHER 1958) The results are reported as means ± standard errors of the means When both hydralazine and sodium nitrite were given in the same experiment the effects of the two drugs were compared by statistically analysing the differences between the effects within each experiment

Results

A *Effect of Hydralazine on Blood Flow and Volume of Forearm*

The effect of a five minute intra arterial infusion of hydralazine on the forearm blood flow was reported in a recent paper (ÅBLAD *et al* 1961) a representative experiment from there being recorded in fig 1A The drug produced an increase in blood flow in the forearm on the side of injection (test arm) but no clear cut effect in the other forearm (control arm) The vasodilatation set in slowly over 10–12 minutes and lasted for several hours The effect of the drug was practically constant from about 12 to 30 minutes after beginning the infusion

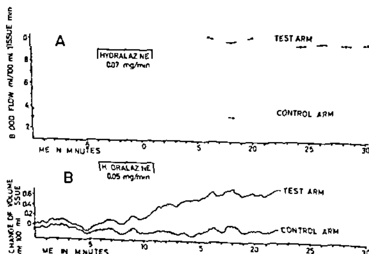


Fig 1 Effect of intra arterial infusion of hydralazine A) on forearm blood flow and B) on forearm volume

Fig 1B shows a representative experiment in which was studied the effect on the forearm volume of infusing hydralazine intra arterially for five minutes The volume of the test arm began to increase 1–2 minutes after beginning the infusion, continued to increase for about 10 more minutes and then levelled off The volume of the control arm did not change appreciably

The results indicate that hydralazine produced an increase in test arm volume co ordinated in time with the effect of the drug on test arm blood flow

B *Effect of Sodium Nitrite on Blood Flow and Volume of Forearm*

Intra arterial infusion of sodium nitrite produced an increase in test arm blood flow (fig 2A) The effect set in immediately after beginning infusion The blood flow increased rapidly during the first five minutes and then almost levelled off When the infusion was stopped, the test arm blood flow immediately began to decrease and had returned to the pre infusion level about 30 minutes later The blood flow in the control arm did not change during the infusion of the drug – Table 1 shows the effects on blood flow obtained in five subjects

Fig 2B shows a representative experiment in which the effect of sodium nitrite on the forearm volume was studied Immediately after beginning infusion the volume of the test arm began to increase After about five

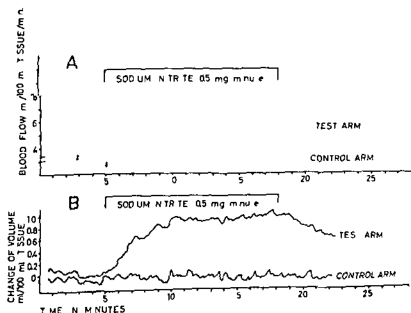


Fig 2 Effect of intra arterial infusion of sodium nitrite A) on forearm blood flow and B) on forearm volume

Effect of intra arter al infusion of sodium nitrite on blood flow in upper part of forearms
Blood flow in ml/m n /100 ml tissue

Exp No	Forearm volume ml		Intra arterial dose of $NaNO_2$ Mg/min	BLOOD FLOW: Before infusion of $NaNO_2$		7 8 minutes after beginning infusion of $NaNO_2$		9 10 minutes after beginning infusion of $NaNO_2$		12–16 minutes after beginning infusion of $NaNO_2$		About 30 m nutes after ending infusion of $NaNO_2$	
	Test arm	Contr arm		Test arm	Contr arm	Test ram	Contr arm	Test arm	Contr arm	Test arm	Contr arm	Test arm	Contr arm
1	680	660	0 53	2 4	2 5	3 9	2 8	5 9	3 7	5 8	3 7	3 3	3 4
2	590	550	0 89	3 8	3 6	9 2	3 7	9 1	3 7	9 7	3 7	4 2	3 9
3	665	650	0 53	1 9	2 0	2 9	2 0	3 2	2 4	3 4	2 4	2 9	2 7
4	820	760	0 36	8 0	6 5	10 9	5 9	11 0	5 9	11 5	6 3	7 2	5 2
5	680	695	0 53	3 1	3 4	5 3	3 5	5 3	3 2	5 3	3 3	3 1	3 2
Mean value s.e m	687 ±37 2	663 ±34 2	0 57 ±0 087	3 8 ±1 09	3 6 ±0 78	6 4 ±1 55	3 6 ±0 65	6 9 ±1 39	3 8 ±0 58	7 2 ±1 48	3 9 ±0 65	4 1 ±0 80	3 7 ±0 43
Mean change in test arm after sodium nitrite s e m						2 6 ±0 77 p<0 05		3 1 ±0 67 p<0 01		3 4 ±0 74 p<0 02		0 3 ±0 33	
Mean change in control arm after sodium nitrite s e.m							0 ±0 15		0 2 ±0 30		0 3 ±0 25		0 1 ±0 39

minutes the volume tended to level off Immediately after the infusion was stopped, the test arm volume began to decrease

The results thus indicate that sodium nitrite also produced an increase in volume of the forearm co ordinated in time with the effect of the drug on the test arm blood flow

C *Quantitative Comparison between the Effects of Sodium Nitrite and Hydralazine on Blood Flow and Volume of Forearm*

The results reported above indicated that it should be possible to evaluate quantitatively the effects of both sodium nitrite and hydralazine on the volume as well as on the blood flow of the forearm in one and the same experiment This was done on six subjects and fig 3 illustrates a representative experiment First the blood flow was measured for about ten minutes (not shown in the figure) and found to be about 3 ml/min /100 ml tissue in both forearms The volume was then recorded continuously, and sodium nitrite was infused in the test arm The drug produced an increase in test arm volume at a rapid rate for the first five minutes and then at a much lower rate About 13 minutes after beginning infusion the volume recording was interrupted and instead blood flow was recorded during continued infusion of the drug About 4 minutes later the infusion of sodium nitrite was stopped, and the blood flow was measured until it was equal in both forearms Then a new period of volume recording was initiated (lower part, fig 3) and hydralazine was infused in the test arm for five minutes This drug produced a gradual increase in test arm volume From the 11 minute after beginning infusion the test arm volume re

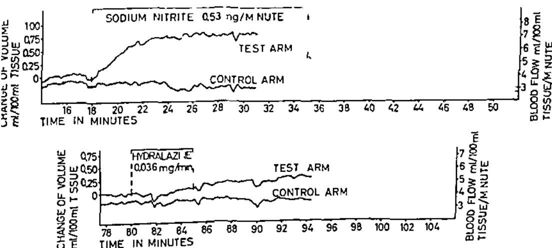


Fig 3 Effect of intra arterial infusion of sodium nitrite (upper section) and hydralazine (lower section) on volume (continuous lines) and blood flow (dashed lines) of upper part of forearms

mained relatively constant About four minutes later the volume re cording was stopped, and blood flow was recorded for about 10 minutes The test arm blood flow was now relatively constant and distinctly higher than before hydralazine was given, whereas the control arm blood flow was essentially unchanged – In this subject both drugs produced about the same increase in test arm blood flow, but hydralazine evoked a much smaller increase in test arm volume than sodium nitrite

The first six experiments in table 2 were performed as just described If the "real' effect of the drugs on the test arm blood flow and volume is calculated by subtracting the changes in the control arm from those in the test arm it is found that hydralazine always evoked a greater increase of blood flow and a smaller increase of volume than did sodium nitrite Statistical evaluation of the differences between the calculated "real' drug effects within each experiment shows that the mean volume increase after hydralazine was 0 27 ± 0 083 ml/100 ml tissue smaller than that after sodium nitrite ($p < 0\ 05$) and that the average increase in blood flow was 1 5 ± 0 77 ml/min /100 ml tissue greater after hydralazine than after sodium nitrite

This difference between the two drugs might have been due to the fact that the hydralazine was always administered after the sodium nitrite To test this possibility hydralazine was given as the first and only drug in five experiments, which were otherwise conducted in the usual manner The mean results of these experiments (table 3) were quantitatively similar to those obtained when hydralazine was given after sodium nitrite On an average the calculated "real' increase in test arm blood flow differed by 0 1 ± 1 12 ml/min /100 ml tissue and in test arm volume by 0 09 ± 0 072 ml/100 ml tissue between the two series

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[illegible] ml/100 ml tissue)

For a further evaluation of the results a calculation was made of the quotient

$$\frac{\text{'real' increase of test arm blood flow}}{\text{"real' increase of test arm volume}}$$

for each drug in all experiments This quotient was for all 11 experiments with hydralazine 11 8 ± 1 22 and for all 8 experiments with sodium nitrite 5 2 ± 0 87 The difference between the two mean quotients was 6 6 ± 1 50, which was statistically significant ($P < 0\ 001$)

Table 2

Effect of intra arterial infusion of sodium nitrite (experiments 6–13) and hydralazine (experiments 6–11) on volume and blood flow of upper part of forearms
Volume changes in ml/100 ml tissue Blood flow in ml/min /100 ml tissue

Exp No	Forearm volume ml		Intra arterial dose of $NaNO_2$ mg/min	Intra arterial dose of hydra lazine mg/min	Blood flow before infusion of $NaNO_2$		SODIUM NITRITE: Volume change 9–10 minutes after beginning infusion		SODIUM NITRITE: Blood flow 12–16 min after beginning infusion		Blood flow before infusion of hydralazine		HYDRALAZINE: Volume change 13–14 minutes after beginning infusion		HYDRALAZINE: Blood flow 18–27 min after beginning infusion	
	Test arm	Contr arm			Test arm	Contr arm	Test arm	Contr arm	Test arm	Contr arm	Test arm	Contr arm	Test arm	Contr arm	Test arm	Contr arm
6	645	660	0 53	0 024	3 5	2 7	0 32	−0 08	3 8	2 4	2 3	2 4	0 25	0 05	4 6	3 3
7	700	680	0 53	0 071	4 9	5 5	0 49	−0 05	7 4	5 3	4 8	4 8	0 58	0 11	12 8	5 0
8	565	610	0 89	0 047	4 2	4 1	0 68	−0 13	6 8	4 5	3 7	3 7	0 08	−0 31	6 3	3 7
9	615	750	0 53	0 036	2 9	3 0	0 74	−0 10	5 3	2 7	2 9	2 8	0 24	−0 01	5 9	2 8

	755	680	0.53	0.047	4.6	3.7	0.62	0.31	7.4	4.1	4.3	3.7	0.39	0.16	9.2	4.3
	800	710	0.89	0.071	2.8	2.6	0.58	−0.01	8.1	3.1	3.7	3.1	0.30	−0.02	8.2	2.6
value exp no 6–11	680	682	0.65	0.049	3.8	3.6	0.57	−0.01	6.5	3.7	3.6	3.4	0.31	0.00	7.8	3.6
	±36.1	±19.2	±0.076	±0.0077	±0.36	±0.45	±0.061	±0.066	±0.66	±0.46	±0.37	±0.35	±0.068	±0.067	±1.20	±0.37
12	810	785	0.36		4.1	5.1	0.31	−0.08	7.2	5.6						
13	835	740	0.53		6.1	5.3	0.15	−0.04	7.2	5.2						
Mean value exp no 6–13	716	702	0.60		4.1	4.0	0.49	−0.02	6.7	4.1						
s e m	±35.3	±19.8	±0.067		±0.39	±0.42	±0.073	±0.049	±0.50	±0.44						

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Effect of intra arterial infusion of sodium nitrite (experiments 6–13) and hydralazine (experiments 6–11) on volume and blood flow of upper part of forearms
Volume changes in ml/100 ml tissue Blood flow in ml/min /100 ml tissue

Exp No	Forearm volume ml		Intra arterial dose of $NaNO_2$ mg/min	Intra arterial dose of hydralazine mg/min	Blood flow before infusion of $NaNO_2$		SODIUM NITRITE: Volume change 9–10 *minutes* after beginning infusion		SODIUM NITRITE: Blood flow 12–16 *min* after beginning infusion		Blood flow before infusion of hydralazine		HYDRALAZINE: Volume change 13–14 *minutes* after beginning infusion		HYDRALAZINE: Blood flow 18–27 *min* after beginning infusion	
	Test arm	Contr arm			Test arm	Contr arm	Test arm	Contr arm	Test arm	Contr arm	Test arm	Contr arm	Test arm	Contr arm	Test arm	Contr arm
6	645	660	0 53	0 024	3 5	2 7	0 32	−0 08	3 8	2 4	2 3	2 4	0 25	0 05	4 6	3 3
7	700	680	0 53	0 071	4 9	5 5	0 49	−0 05	7 4	5 3	4 8	4 8	0 58	0 11	12 8	5 0
8	565	610	0 89	0 047	4 2	4 1	0 68	−0 13	6 8	4 5	3 7	3 7	0 08	−0 31	6 3	3 7
9	615	750	0 53	0 036	2 9	3 0	0 74	−0 10	5 3	2 7	2 9	2 8	0 24	−0 01	5 9	2 8

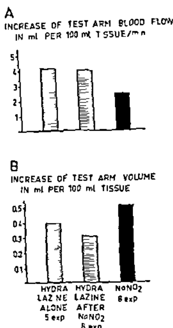


Fig 4 Mean calculated 'real' effects of hydralazine and sodium nitrite on test arm blood flow (A) and total volume (B)

On the whole the results thus indicate that there was a quantitative difference between the effects of the two drugs on blood flow and volume of forearm The mean results are graphically illustrated in figure 4

D *Quantitative Comparison between the Effects of Sodium Nitrite and Hydralazine on Volume and Venous Pressure of Forearm*

In 4 experiments the volume changes occurring in the upper part of the forearms were recorded continously in the usual manner, and continuous recordings of the subcutaneous venous pressure in the forearms were also made In each experiment an intra arterial infusion of sodium nitrite was first given, about 30 minutes after interruption of this infusion, hydralazine was infused The drugs were given in about the same doses as in the experiments reported above

Before the drugs were given, the mean venous pressure was about 5 cm H_2O in both forearms (range 3–8 cm), and there was on an average no significant difference between the simultaneously recorded values in the two forearms When sodium nitrite or hydralazine was administered intra-arterially, venous pressure in the test arm increased, and the change

Table 3

Effect of intra arterial infusion of hydralazine on volume and blood flow of upper part of forearms
Volume changes in ml/100 ml tissue Blood flow in ml/min /100 ml tissue

Exp No	Forearm volume ml		Dose of hydralazine mg/min	*Blood flow* before infusion of hydralazine		HYDRALAZINE: Volume change 13–14 minutes after beginning infusion of hydralazine		HYDRALAZINE: Blood flow 18 27 minutes after beginning infusion of hydralazine		HYDRALAZINE: Blood flow 28–37 minutes after beginning infusion of hydralazine		HYDRALAZINE: Blood flow 38–47 minutes after beginning infusion of hydralazine	
	Test arm	Contr arm		Test arm	Contr arm	Test arm	Contr arm	Test arm	Contr arm	Test arm	Contr arm	Test arm	Contr arm
14	740	720	0 047	3 1	2 7	0 28	−0 25	8 5	3 6	8 8	3 5	9 2	3 7
15	730	645	0 047	3 7	3 3	0 29	−0 07	9 0	3 8	9 2	3 2	7 8	3 3
16	615	660	0 024	2 4	2 3	0 45	0 25	4 5	2 4	4 6	2 4	5 1	2 5
17	860	830	0 047	5 1	6 0	0 64	0 19	10 3	5 4	9 7	4 3	9 7	4 1
18	715	690	0 024	4 9	4 4	0 23	−0 21	8 6	4 4	8 4	3 9	8 5	4 1
Mean value s e m	732 ±39 0	709 ±32 9	0 038 +0 0056	3 8 ±0 52	3 7 [illegible]	0 38 [illegible]	−0 02 [illegible]	8 2	3 9	8 1	3 5	8 1	3 5

importance for the volume increase due to hydralazine than for that due to sodium nitrite

The extravascular fluid volume can be changed by vaso active agents through a change of the mean capillary hydrostatic pressure (MELLANDER 1960) The extravascular fluid volume is also controlled by the outflow in the lymph vessels The lymph outflow was probably completely blocked during our volume recordings, as RUSHMER & IRISAWA (1960) showed on the hind leg of the dog that during venous occlusion with a proximal cuff the lymphatic pressure rose slowly and stayed far below the venous pressure

It is impossible to be certain how hydralazine and sodium nitrite changed the extravascular volume in our experiments As it was necessary to have information on this point before the effects of the drugs on the capacitance vessels could be evaluated, we have studied this problem further on anaesthetized cats by a more elaborate technique (ÅBLAD & MELLANDER 1963) The results agreed well with those in man, in that hydralazine produced a greater dilatation in resistance vessels but a smaller increase in total volume than did sodium nitrite Further, it was found that hydralazine produced an *increase*, whereas sodium nitrite produced *no clear change* in extravascular fluid volume – We have also performed a study in man of the blood volume changes induced by intra venously administered hydralazine or sodium nitrite (unpublished observations), the results obtained showed a difference between the effects of the two drugs that correlated well with the differential effects on extravascular volume found in the cat It is intended to discuss the cause of this difference later when the results of the study on blood volume are reported

Because of the findings just described, it seems possible to draw a clearer conclusion from the results obtained in this investigation The quantitative difference observed between the effects of hydralazine and sodium nitrite on blood flow and total volume of forearm was probably due to the fact that *the dilating action of hydralazine was more pronounced on the resistance vessels and less pronounced on the capacitance vessels than was that of sodium nitrite* The "spasmolytic" action of the two drugs on the capacitance vessels possibly differed more than the values for total volume increase indicate, partly because the pressure induced distention of the post arteriolar vessels might have been of greater importance for the total volume increase after hydralazine, partly because an increase in extravascular volume might have produced some of the total volume increase after hydralazine

The difference found between the actions of hydralazine and sodium nitrite on the resistance and capacitance vessels may in part explain why

followed the same time course as the volume increase, which for drug was similar to that recorded in the experiments already desc The venous pressure of the control arm did not on average cl significantly during the drug infusions, but in the individual experi there occurred slight and varying changes These changes, which considered not to be related to the drug infusion, probably also occ in the test arm and interfered with the drug-induced changes there "real" effect of the drugs on the venous pressure of the test arm in experiment was estimated, in a similar way to the effects on test volume and blood flow, by subtracting the change occurring ir control arm from that in the test arm

In every experiment hydralazine produced a greater increase in ve pressure than did sodium nitrite in the test arm, if the effects were (pared at a time when the two drugs had produced about the same inc of the forearm volume The mean calculated "real" increase of the arm volume, produced by hydralazine about 11–14 minutes after be ning infusion, was 0 45 ± 0 130 ml/100 ml tissue, and that produce sodium nitrite about 4–7 minutes after beginning infusion of this (was almost the same, 0 46 ± 0 138 ml/100 ml tissue (difference 0 0 0 009 ml/100 ml tissue) The simultaneously recorded "real" increas venous pressure produced by hydralazine was on average 1 6 ± 0 28 H_2O, whereas that produced by sodium nitrite was on average 0 0 31 cm H_2O The difference was statistically significant (0 7 ± 0 13 H_2O, $P < 0\ 02$)

These results indicate that there was a quantitative difference betw the effects of the two drugs on volume and subcutaneous venous press in the forearm

Discussion

Volume changes in the forearm may be due partly to a change vascular capacity with consequent change in blood volume of the forear partly to a change in amount of extravascular fluid

The vascular blood capacity in the forearm could have been increas by hydralazine and sodium nitrite in two ways Part of the increase mig be due to reduced tone of the smooth muscle cells in the capacitan vessels, the other part of the increase being due to a pressure-induc distention of the post-arteriolar vessels, the drugs dilating the resistan vessels with a consequent reduction in arterio venous pressure gradie which lead to an increase in venous pressure If the pressure chang recorded in a subcutaneous vein were representative for the whole for arm this passive distention should have been of comparatively great

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The difference found between the actions of hydralazine and sodium nitrite on the resistance and capacitance vessels may in part explain why

followed the same time course as the volume increase, which for each drug was similar to that recorded in the experiments already described The venous pressure of the control arm did not on average change significantly during the drug infusions, but in the individual experiments there occurred slight and varying changes These changes, which were considered not to be related to the drug infusion, probably also occurred in the test arm and interfered with the drug-induced changes there The "real" effect of the drugs on the venous pressure of the test arm in each experiment was estimated, in a similar way to the effects on test arm volume and blood flow, by subtracting the change occurring in the control arm from that in the test arm

In every experiment hydralazine produced a greater increase in venous pressure than did sodium nitrite in the test arm, if the effects were compared at a time when the two drugs had produced about the same increase of the forearm volume The mean calculated "real" increase of the test arm volume, produced by hydralazine about 11–14 minutes after beginning infusion, was 0 45 ± 0 130 ml/100 ml tissue, and that produced by sodium nitrite about 4–7 minutes after beginning infusion of this drug was almost the same, 0 46 ± 0 138 ml/100 ml tissue (difference 0 01 ± 0 009 ml/100 ml tissue) The simultaneously recorded "real" increase in venous pressure produced by hydralazine was on average 1 6 ± 0 28 cm H_2O, whereas that produced by sodium nitrite was on average 0 9 ± 0 31 cm H_2O The difference was statistically significant (0 7 ± 0 13 cm H_2O, $P < 0\ 02$)

These results indicate that there was a quantitative difference between the effects of the two drugs on volume and subcutaneous venous pressure in the forearm

Discussion

Volume changes in the forearm may be due partly to a change in vascular capacity with consequent change in blood volume of the forearm, partly to a change in amount of extravascular fluid

The vascular blood capacity in the forearm could have been increased by hydralazine and sodium nitrite in two ways Part of the increase might be due to reduced tone of the smooth muscle cells in the capacitance vessels, the other part of the increase being due to a pressure-induced distention of the post-arteriolar vessels, the drugs dilating the resistance vessels with a consequent reduction in arterio venous pressure gradient, which lead to an increase in venous pressure If the pressure changes recorded in a subcutaneous vein were representative for the whole forearm, this passive distention should have been of comparatively greater

Rushmer, R F. & A Irisawa Relationship between lymphatic and venous pressure in leg of dog *Am J Physiol* 1959, 196, 495–498

[illegible]

Åblad, B : Site of action of hydralazine and dihydralazine in man *Acta pharmacol et toxicol* 1959, 16, 113–128

Åblad, B , G Johnsson & M Henning The effect of intra arterially administered hydralazine on blood flow in the forearm and hand *Acta pharmacol et toxicol* 1961, 18, 191–198

[illegible]

nitrite and acetylcholine on resistance and capacitance blood vessels and capillary filtration in skeletal muscle in the cat *Acta physiol Scand* 1963, in press

the two drugs produce different haemodynamic effects in the intact circulation after systemic administration It is proposed to discuss this subject on a later occassion

Summary

Hydralazine or sodium nitrite was infused in low dosage into a brachial artery and the effects on blood flow and volume of the upper part of the forearm were studied separately Hydralazine, which was infused for five minutes, produced a gradual increase in both blood flow and volume of the forearm The effect reached its maximum about 12 minutes after beginning infusion and was then relatively constant for several minutes Sodium nitrite also evoked an increase in both measurements, the rate of increase was rapid for the first five minutes of the infusion and then proceeded at a much slower rate until the end of the infusion A comparison of the effects of the two drugs showed that hydralazine evoked a greater increase in blood flow but a smaller increase in volume of forearm than did sodium nitrite The relative importance of various mechanisms for the volume increase is discussed It is concluded that the results indicate the spasmolytic action of hydralazine – compared with that of sodium nitrite – to be more pronounced on the resistance vessels and less pronounced on the capacitance vessels

Acknowledgement

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REFERENCES

Fisher, R A Statistical methods for research workers Edinburgh 1958 pp 356

Folkow, B The efferent innervation of the cardiovascular system *Verh* [illegible]

[illegible] E A [illegible] in IV

[illegible] ent of [illegible] 1958

Mellander, S Comparative studies [illegible] monal control of resistance and capacitance blood vessels in the cat *Acta physiol Scand* 1960, 50 suppl 176, 1–86

Moyer, J H, C A Handley & R A Huggins Some pharmacodynamic effects of 1 hydrazinophtalazine (C 5968) with particular reference to renal function and cardiovascular response *J Pharmacol* 1951 103 368–376

In the study reported below, the hepatotoxic effects of inhaled trichloroethylene, tetrachloroethylene and chloroform on the mouse were examined The object was to find the smallest doses of the substances producing signs of liver damage The effect on the organ was evaluated by (i) histological examination, (ii) determination of the substances in the liver that are soluble in a mixture of acetone and hexane (fat extraction), and (iii) determination of the ornithine carbamoyl transferase activity in serum (S-OCT) This method is highly sensitive and specific for liver damage (REICHARD & REICHARD 1958, REICHARD 1960, REICHARD 1962) Chloroform was included in the study for the purpose of comparison, since it is known to cause liver damage in animals and man

Materials and Methods

Solvents Trichloroethylene (<0 2% impurities) Tetrachloroethylene (<0 5% trichloroethylene and <0 5% other impurities) Chloroform, analytical grade

Animals Th[illegible]

mean weight [illegible]

percentage co[illegible]

powdered ski[illegible] milk [illegible]%, milled whole oats 20%, dry brewer s yeast 1,5%, milled whole barley 10%, wheat germ 3% soybean flour 10% iodinised salt 0 3%, powdered meat 8%, vitamins

Exposure The mice were exposed to the agents for four hours in a fume chamber of about 15 litres volume, which was supplied with a constant current of air via a vaporizing unit The air in the chamber was changed about once a minute The solvent was injected into th[illegible]

syringe The c[illegible]

solvent was su[illegible]

Examinatio[illegible] liver was weighed immediately after dissection and cut in two one half being used for the fat extraction and the other for histological examination

(a) Histological methods After the liver had been fixed in neutral 10% (v/v) formalin frozen and paraffin sections were prepared The former were stained with Scharlach R haematoxylin, and the latter with haematoxylin-eosin, haematoxylin by van Gieson s method, and toluidin blue basic fuchsin at pH 4 by Romhanyi s method (*KELÉNYI 1956*)

It was originally intended to carry out a more comprehensive examination of the histological changes, but the reproducibility of such findings was poor, and the evaluation was therefore limited to necrosis and degrees of fat infiltration classified as

(i) no infiltration

(ii) moderate infiltration of fat Fatty degeneration involving [illegible]

(ii[illegible]

Acta pharmacol et toxicol 1963, 20, 16–26

From the National Institute of Public Health, Department of Occupational hygiene (Head A Ahlmark), Karolinska institutet, the Institute of Hygiene (Head L Friberg) King Gustaf V Research Institute (Head G Birke) Karolinska Hospital, the Department of Medicine (Head H Lagerlof) and the Department of Pathology (Head Å Wilton), *Stockholm Sweden*

Hepatotoxicity of Inhaled Trichloroethylene, Tetrachloroethylene and Chloroform. Single Exposure

By

Bengt Kylin, Hans Reichard, Istvan Sümegi & Sven Yllner

(Received December 6 1962)

The literature on the toxicology of tri- and tetrachloroethylene, two degreasing and detergent agents widely used in industry, has been reviewed by VON OETTINGEN (1955)

About trichloroethylene, apart from deaths due to its narcotic action, there have been few reports of organic damage from exposure to it, nor is it certain that even in these cases, which have chiefly involved liver damage, the cause lay with trichloroethylene and not with some other agent Experiments on laboratory animals have yielded rather divergent results, though some workers have found liver alterations, usually fatty degeneration, after fairly long exposure of various species to trichloroethylene, others have found no such effect (ANDERSSON 1957)

About tetrachloroethylene, the effect of inhalation has received little attention, and as for trichloroethylene, there are only a few reported instances of organic damage (COLER & ROSSMULLER 1953, LOB 1957) Clear fatty degeneration of the liver has been produced in animals in a few studies (CHRISTENSEN & LYNCH 1933, ROWE, MCCOLLISTER, SPENCER, ADAMS & IRISH 1952) and in one study mild cirrhosis has been observed (ROWE *et al* 1952)

Determinations of the maximum exposure tolerated by the rat without death or organic damage have shown tetrachloroethylene to be the more toxic of the two agents, but only slightly so (ROWE *et al* 1952, ADAMS, SPENCER, ROWE, MCCOLLISTER & IRISH 1951) The contrary result was obtained by PLAA, EVANS & HINE (1958), who examined the relative hepatotoxicity of some chlorinated hydrocarbons in the mouse by determining the prolongation of pentobarbital anaesthesia after subcutaneous injection of the solvent This view is shared by MOESCHLIN (1956)

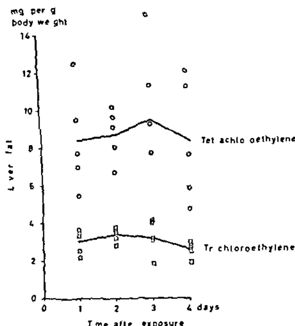


Fig 1 The amount of liver fat found at different times after exposure to 3200 p p m trichloroethylene or tetrachloroethylene

Neither the pilot studies nor the investigations mentioned give any exact indication of when, during the first three days after exposure, the peak increase in S-OCT activity is to be expected In our study it was therefore decided to perform the examination on mice killed one or three days after exposure

Results

Histological Findings (Fig 2)

1 *Trichloroethylene* – None of the mice killed one or three days after exposure to a concentration of 1600 p p m differed from the controls in the extent of liver damage At 3200 p p m the frequency of moderate infiltration of fat on both days was slightly greater than at the lower concentration, there was, however, no clear difference between the exposed and control groups in this condition

2 *Tetrachloroethylene* – At a concentration of 200 p p m a moderate infiltration was observed in most of the animals of the one-day group, but there was no evident increase by the third day At 400 p p m most of the mice in the one and three-day groups displayed moderate to massive infiltration At higher doses the infiltration was more pronounced, but even in the highest (1600 p p m) there was no evidence of cell necrosis

(b) Fat extraction The liver was cut into small pieces and extracted overnight with equal volumes of acetone and hexane in a Soxhlet apparatus The non volatile extract which was not analysed for its constituent components was classed as liver fat

Determination of S OCT activity The mice were beheaded and as much of the blood as possible was collected The serum amounting as a rule to about 0 2 ml was diluted to 1 ml with physiological saline and cooled immediately to −20 C

The S OCT activity was determined by incubating the diluted serum with ^{14}C citrullin in an arsenate buffer by the method of REICHARD & REICHARD (1958) The amount of liberated $^{14}CO_2$ is proportional to the OCT activity The results were expressed in µmoles $^{14}CO_2$ per ml undiluted serum

Tissue homogenates Homogenates of tissues were prepared from animals killed by beheading The method by which the specimens were treated has been described elsewhere (REICHARD 1960)

Pilot studies

S-OCT activity In a pilot study carried out to find how long after exposure an increase in S-OCT activity reached its maximum, a number of mice were exposed to 3000 p p m of trichloroethylene, tetrachloroethylene or chloroform for 4 hours The animals were killed one hour and 1, 2, 4, 8 and 16 days after exposure Each group consisted of 5 mice, the total number, including controls, was 95

Trichloroethylene One mouse of the 5 killed 24 hours after exposure showed a greatly elevated S-OCT activity and 2 mice of the 2 day group somewhat high values

Tetrachloroethylene On the first, second and fourth days occasional somewhat elevated values were noted

Chloroform All the mice showed greatly raised values on the first 4 days after exposure

Liver fat A comparative study was made of the amounts of fat extract from the liver soluble in the acetone hexane mixture at different times after exposure to 3200 p p m of trichloroethylene or tetrachloroethylene The quantity was considerably higher for tetrachloroethylene than for trichloroethylene (Fig 1), For neither agent was the difference between days statistically significant

Comments In an examination of the S OCT activity in man after obstetric anaesthesia with chloroform, the maximum increase has been recorded on the third day after exposure (REICHARD, WIQUIST & YLLNER 1960) In the dog to which carbon tetrachloride was supplied through a stomach tube the peak occurred on the second day (REICHARD 1959) Rabbits inhaling this agent showed peak values for S-GPT and S-LDH activities 24 hours after exposure (FRANKE, GAERTNER, KOSSUTH & MILCH 1957)

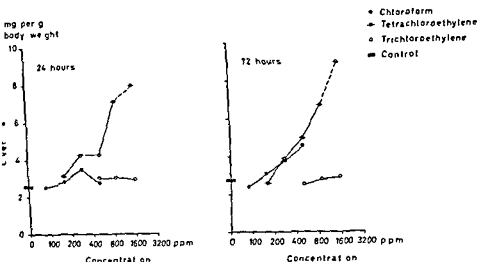


Fig 3 Liver fat related to concentration of agent
Each dot represents the mean of 10 animals (The value of 3200 p p m tetrachloroethylene is taken from pilot investigations)

Tetrachloroethylene There was a marked increase in the amount of liver fat with concentration, the amount of fat being significantly greater than for the control groups at 400 p p m and above

Chloroform After one day there was an increase with concentration up to 400 p p m and after 3 days up to 800 p p m

S OCT Activity

This enzyme in the mouse is localized chiefly in the liver (table 1) The activities in the homogenates of the small intestine and the colon were only about 3 and 0 5%, respectively, of that of the liver Other organs showed negligible activities In serum from the controls the corresponding values were 0 15 ± 0 9 µmoles

The mice killed after exposure to chloroform for 24 hours showed a significantly higher S–OCT activity than the controls for the concentrations 200, 400 and 800 p p m , 3 days after exposure significant differences were recorded only for the 800 p p m group (figs 4 a and b) Tri- and tetrachloroethylene did not produce any increase in the S–OCT activity

Relation between S OCT Activity and Liver Cell Necrosis

The relationship between the histologically demonstrable liver-cell necrosis and the increase in S–OCT activity is illustrated in table 2 The

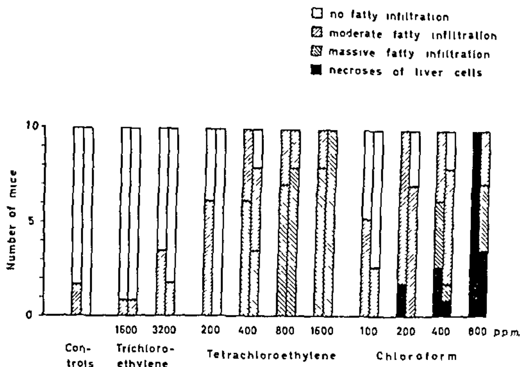


Fig 2 Number of mice with different degrees of fatty infiltration and with cell necroses in the exposed groups The left column represents the results 24 hours, and the right 72 hours, after exposure

3. *Chloroform.* – With a concentration of 100 p.p.m. moderate infiltration was noted in a larger number of the mice killed one day after exposure than of the controls. At 200 p.p m and above, the extent of the alterations increased with concentration and was more pronounced after one day than three.

Thus, to judge from the histological picture the smallest concentrations of the different agents to produce more severe alterations in the exposed group than was observed in the controls were as follows:

	Trichloro- ethylene	*Tetrachloro-* ethylene	*Chloro-* form
One day after exposure	1600–3200	<200	<100
Three days.	>3200	200–400	100–200

On this basis the hepatotoxic effects of trichloroethylene, tetrachloroethylene and chloroform are in the approximate ratios *1 : 10 · 20.*

Amount of Extractable Liver Fat (fig 3)

Trichloroethylene. In neither the one- nor the three-day groups was any increase in liver fat recorded, whatever the concentration.

F g 4 a and b Orn thine carbamınoyl transferase actıv ty ın serum
ın the d fferent exposure groups

Table 1

Activities of ornithine carbaminoyl transferase in serum and various organs

Organ	Number of mice	Mean OCT (µmoles)*)	Range (µmoles)
Liver	5	5200	2300–9100
Small intestine	5	160	96–200
Colon	6	29	14–51
Lung	6	2 0	1 3–2 9
Kidney	5	1 2	0 6–2 4
Muscle	6	1 0	0 3–2 7
Spleen	6	0 7	0 1–1 5
Brain	5	0 2	0 03–0 5
Heart	5	0 2	0 1–0 5
Serum	25	0 15	0 00–0 40

*) Expressed as µmoles of $^{14}CO_2$ per g of tissue

class interval in enzyme values is the difference between the extreme values for the unexposed animals (that is 0 0–0 40 µmoles)

High S–OCT activities were extremely common when there was histologically demonstrable liver cell necrosis In the absence of such necrosis, slightly raised values (0 41–0 80 µmoles) were recorded in about 9 % of the mice and greatly raised one (> 1 21 µmoles) in about 3 % As has been pointed out above, necrosis was noted only in the groups exposed to chloroform

Discussion

In this study of the toxic action of chlorinated aliphatic hydrocarbons, special attention has been paid to liver damage, since that organ seems to be the most susceptible to these solvents (VON OETTINGEN 1955) It spite of extensive study of cytological and biochemical changes in the liver, the nature of the toxic action remains uncertain Among the familiar pathologic changes in the liver there are two that are easily recognized histologically, namely the accumulation of fat and necrosis of the liver cells

Table 2

Relationship between S OCT activity and liver cell necrosis 200 specimens from exposed mice

	S–OCT activity (µmoles)				Total
	0 00–0 40	0 41–0 80	0 81–1 20	>1 20	
Liver cell necrosis	1	0	1	19	21
No necrosis	156	16	2	5	179

lethal doses of these substances and their narcotic properties (FRIBERG, KYLIN & NYSTRÖM 1953) have shown that tetrachloroethylene has a slightly greater toxicity than trichloroethylene The study described here showed that in hepatotoxicity, tetrachloroethylene was the more dangerous of the two, although no more severe damage than fatty infiltration was noted The possibility of more serious liver injury through long or repeated exposure to these substances has been examined, and it is hoped to report the results later

Summary

The hepatotoxic effects of four hours' exposure to trichloroethylene, tetrachloroethylene and, for comparison, chloroform were studied in mice The effect on the organ was evaluated by histological examination, determination of the liver fat and determination of the ornithine carbamoyl transferase activity in serum (S–OCT)

Trichloroethylene did not produce any marked signs of liver damage, even in doses up to 3200 p p m

With tetrachloroethylene, moderate fat infiltration was observed already at a concentration of 200 p p m and the amount of liver fat was raised at 400 p p m

Chloroform produced moderate fat infiltration at a concentration of 100 p p m and the amount of liver fat was raised at 400 p p m

Necrosis as well as increased S–OCT activity was only observed after exposure to chloroform

The study showed that the presence of liver cell necrosis is almost unvariably associated with a marked rise in S–OCT activity

The hepatotoxic effects of the three substances examined, assessed on the basis of the fat infiltration, were approximately in the ratio 1 10 20 for tri and tetrachloroethylene and chloroform, respectively

REFERENCES

Adams E M H C Spencer V K Rowe D D McCollister & D D Irish Vapor Toxicity of Trichloroethylene Determined by experiments on Laboratory Animals *Arch Ind Hyg Occup Med* 1951 4 469–481

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In man ornithine carbamoyl transferase occurs mainly in the liver, and only small amounts are present in the small intestine (REICHARD 1960) Since, as shown in this study, the distribution of S-OCT is much the same in the mouse as in man, the determination of the S-OCT activity is probably no less sensitive and specific a test for acute liver cell damage in the former than in the latter (REICHARD 1962)

The study has shown that the presence of liver cell necrosis is almost invariably associated with a marked rise in S-OCT activity The marked increase noted when there was no evidence of liver cell damage may have been due to inadvertent selection of the sections from non-representative parts of the organ; another possibility is that ornithine carbamoyl transferase leaked out of vital liver cells also. Support for this theory is found in clinical observations on S-OCT (REICHARD 1962) and other enzymes (SCHLANG & KIRKPATRICK 1961) and the results of animal experiments (DINMAN, FOX, FRAJOLA & RABOR 1962) Whatever the explanation, there was a high correlation between the increase in S-OCT and liver cell necrosis

As reported earlier (KYLIN, REICHARD, SÜMEGI & YLLNER 1962), the extent of fat infiltration judged on the sections was in close agreement with the quantity of extractable total lipids in the liver The hepatotoxic effects for the three substances examined, assessed on the basis of fat infiltration, were approximately in the ratios 1 10 20 for tri- and tetrachloroethylene and chloroform, respectively The same relationship between the liver toxicity of trichloroethylene and chloroform was reported by JONES, MARGOLIS & STEPHEN (1958) for mice to which different amounts of the solvents mixed in olive oil had been administered orally

If an increase in liver fat is indicative of liver damage, it would seem from the present findings that it may be more dangerous to inhale tetrachloroethylene than trichloroethylene

Although extensive infiltration of fat in the liver may affect its function and fatty degeneration of the liver may be an early stage of cirrhosis (POPPER 1961), it is uncertain whether the fatty degeneration observed in the mice was a preliminary to permanent injury or only a temporary manifestation

Although one must bear in mind that there is often a considerable disparity between the effects of toxic substances in animals and in man, the results of this study indicate the need for careful deliberation about use of tri- and tetrachloroethylene as a matter of occupational hygiene Though there are wide differences in opinion about the toxicity of the vapour of both these substances (ROWE, MCCOLLISTER, SPENCER, ADAMS & IRISH 1952, ADAMS, SPENCER, ROWE, MCCOLLISTER & IRISH 1951, PLAA, EVANS & HINE 1958, MOESCHLIN 1956), studies at this Institute on the

lethal doses of these substances and their narcotic properties (FRIBERG, KYLIN & NYSTRÖM 1953) have shown that tetrachloroethylene has a slightly greater toxicity than trichloroethylene The study described here showed that in hepatotoxicity, tetrachloroethylene was the more dangerous of the two, although no more severe damage than fatty infiltration was noted The possibility of more serious liver injury through long or repeated exposure to these substances has been examined, and it is hoped to report the results later

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REFERENCES

Adams E M H C Spencer V K [illegible]

And [illegible]

Chri [illegible]

Cole [illegible] [illegible]oethylene exposure in a small Industry *Arch Ind Hyg Occup Med* 1953 8 227 233

Dinman, B D, C F Fox W S Frajola & A Rabor Serum enzyme and B 12 changes in CCl_4 hepatotoxicity *Arch Env Health* 1962, 4 968–182
Franke, H D, P L Gaertner, L C Kossuth & L J Milch Toxic Vapor Inhalation and Serum Enzyme Levels *Texas Rep Biol & Med* 1957 15, 868–873
Friberg L, B Kylin & A Nyström Toxicities of Trichlorethylene and Tetrachlorethylene and Fujiwara s Pyridine Alkali Reaction *Acta pharmacol et toxicol* 1953, 9, 303–312
Jones, W M, G Margolis & C R Stephen Hepatoxicity of inhalation anaesthetic drugs *Anesthesiology* 1958 19, 715–723
Kelènyi, G Histological determination of the isoelectric point *Kisèrletes Orvostudomàny* 1956, 192, 192–195
Kylin B, H Reichard, I Sümegi & S Yllner Hepatotoxic Effect of Tri and Tetrachloroethylene on Mice *Nature* 1962, 193 395
Lob, M Les dangers du perchloréthyléne *Arch Gewerbepath Gewerbehyg* 1957, 16 45–52
Moeschlin, S Klinik und Therapie der Vergiftungen Stuttgart 1956
von Oettingen W F The Halogenated Hydrocarbons, Toxicity and Potential Dangers, Washington, 1955
Plaa G L, E A Evans & C H Hine Relative Hepatotoxicity of seven Halogenated Hydrocarbons *J Pharmacol exp Therap* 1958, 123, 224–229
Popper, H Morphologic and biochemical aspects of fatty liver *Acta Hepato Splenologica* 1961, 8 279–292
Reichard, H Ornithine Carbamoyl Transferase in Dog Serum on Intravenous Injection of Enzyme, Choledochus Ligation and Carbon Tetrachloride Poisoning *J Lab & Clin Med* 1959, 53, 417–425
Reichard H Ornithine Carbamoyl Transferase Activity in Human Tissue Homogenates *J Lab & Clin Med* 1960 56 218–221
Reichard H Serum Ornithine Carbamoyl Transferase Activity in Man A Highly Specific Test of Liver and Biliary Tract Involvement Observation on 695 Patients *Acta Med Scand* 1962 172 In press
Reichard, H & P Reichard Determination of Ornithine Carbamoyl Transferase in Serum *J Lab & Clin Med* 1958 52, 709–717
Reichard, H N Wiquist & S Yllner Chloroform Anaesthesia in Obstetrics Its Effect on the Serum Activity of Ornithine Carbamoyl Transferase Glutamic Oxalacetic Transaminase and Glutamic Pyruvic Transaminase *Acta Obst et Gynec Scandinav* 1960, 120 251–257
Rowe, V K, D D McCollister, H C Spencer E M Adams & D D Irish Vapor Toxicity of Tetrachlorethylene for laboratory Animals and human subjects *Arch Ind Hyg Occup Med* 1952 5 566–579
Schlang H A & C A Kirkpatrick The effect of physical exercise on serum transaminase *Am J Med Sci* 1961, 242 338 341

Acta pharmacol. et toxicol. 1963, 20, 27–38

From the Research Laboratories of the State Alcohol Monopoly (Alko),
Helsinki, Finland
and Department of Zoology, University of Turku, Turku, Finland

Studies on the Mechanism of Stress-induced Reduction of Alcohol Intoxication in Rats

By

Henrik Wallgren and Rauno Tirri

(Received November 6, 1962)

It is common knowledge that fear, trauma and so on may have a sobering effect on human subjects under alcohol intoxication. Observations made in these laboratories, showing that shock reduces ethyl alcohol intoxication in rats, prompted LEIKOLA (1962) to study the effect of vigorous physical exercise on the level of intoxication. He found that after low or moderate doses of alcohol, there was a marked improvement in performance of a simple functional test after stress, although after intraperitoneal injection the blood levels of alcohol remained unchanged. Large oral doses of alcohol (5 mg/g) in combination with exercise resulted, however, in complete exhaustion of the animals.

An understanding of the mechanism involved in the reduction of alcohol intoxication after stress would have relevance to both a general knowledge of the physiology of intoxication and to its practical application. LEIKOLA (1962) suggested as a crude first approximation that the effect might be due to adrenocortical hormones, perhaps acting directly at the neuronal level in the central nervous system, or to an effect on the reticular activating system, possibly mediated by release of adrenaline. In this report it is shown that hypophysectomy has no influence on the effect of swimming on intoxication, indicating that ACTH stimulated release of adrenocortical hormones is not involved. This and other results described below suggest that the main reason for the improvement of performance after stress is an arousal reaction.

Material and Methods

The animals used were mainly male rats, approximately 4 months old, from the laboratory stock but in one experiment immature 11-week-old [illegible]: animals were housed in groups of 7–10 indi-

viduals in metal cages with grid floors. They were fed on a stock ration made up in the laboratory, and water was available *ad libitum*. Animals obtained from Lääketehdas Orion Oy Pharmaceutical manufactures (Helsinki) were kept for one week in the laboratory before they were used for experiments.

Alcohol was always administered intraperitoneally as a 10% (w/v) solution in saline. The tilted plane technique (ARVOLA, SAMMALISTO & WALLGREN 1958, see also FRIEDMAN & INGALLS 1960; WALLGREN, ARVOLA & SAMMALISTO 1960) was used for assessing the state of intoxication. In the tests, the rats are placed facing up the slope of an inclined plane The plane has a rough surface (plywood covered with bronze netting, ca 22 mesh/cm) to which the rats can cling, and the angle at which the animals begin to slide is recorded In the experiments reported here, the animals were tilted twice at each test, and the sum of the resulting angles recorded The results have been expressed as percentages of the performance immediately before injection. It should be noted that reduction of the performance to 45–50% of the initial value represents the lower limit measurable with the test, indicating that the animal is no longer capable of active movement.

The animals were tested 4–5 times on the day before the experiments were begun, in order to accustom them to the handling involved. In the experiments themselves, closed-deck procedures were applied. Pre-treatment of the animals and recording of the sliding angles were not performed by the person testing The initial value was obtained immediately before injection of alcohol or other substances The animals were then tested at 15 min intervals, usually for two hours The results are given as averages ± their standard deviations unless otherwise stated

The pharmacological agents used were. alcohol (absolute alcohol AaS from Alko, for spectrometric purpose), adrenaline from Oy Medica Ab, Helsinki, amphetamine [illegible] were drawn from [illegible] GMBH's, Mann- [illegible]

Experiments and Results

Experiment 1: Hypophysectomy and the Stress Reaction

The hypophyses were removed from 12 animals, a sham operation being performed on 12 control animals. The technique of operation was as described in expt. 48 in D'ARMOUR & BLOOD (1954) After the operation the animals were given free access to 5% glucose solution and 2% NaCl solution as well as to water The success of the hypophysectomy was checked by weighing the animals regularly (weights at operation averaged ca 320 g; three weeks later hypophysectomized rats had lost 45 g and sham-operated had gained 13 g on an average) and after the experiments by inspection.

Twenty days after operation, the performances of the operated and sham-operated animals after injection of 2 mg alcohol/g body weight were

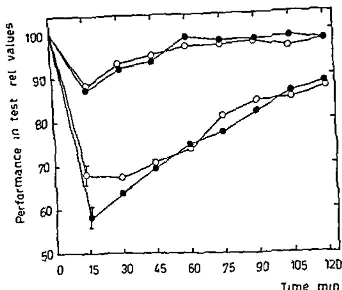


Fig 1 Time course of performance in tilted plane test of hypophysectomized (●) and sham-operated (○) rats after injection of 2 mg alcohol/g i p Lower curves control test upper ones after swimming for 10 min Each point represents the average for 12 rats Standard error of the mean is ind cated by vertical bars for points found to differ significantly

compared There was no difference between the groups (fig 1) except at the first testing 15 minutes after injection at which point the performance of the sham operated animals was significantly better ($P < 0\ 025$, Student's t test)

Three days later, the effect of swimming stress was tested Each rat had a weight of 1 5 g/100 g of its body weight attached to its tail and was allowed to swim in water at 34–35° for 5 minutes immediately before and immediately after injection of alcohol (2 mg/g) Blood samples were taken 45 min after injection The performance of both groups as shown in fig 1 improved greatly after stress, the effect of the alcohol being reduced to about 40% of what it was in the control test without swimming The effect of swimming was highly significant ($P < 0\ 001$) in each

There was an apparent slight but statistically insignificant difference between the blood alcohol levels of sham operated (0 171 ± 0 032%) and hypophysectomized rats (0 150 ± 0 033%) This difference may have been due to the smaller proportion of fat in the hypophysectomized animals

Later determinations of the blood alcohol level of unstressed animals 45 min after injection (expt 2 p 30) gave the values 0 204 ± 0 022% for a group (10 rats) given 2 mg alcohol and 5 μg amphetamine/g body weight, and 0 198 ± 0 032% for a control group (10 rats) receiving alcohol only

Evaluation by Student's t test of the difference between all blood alcohol values obtained in experiment 1 and in experiment 2 shows that it was highly significant ($P < 0\ 001$) Some delay in absorption after swimming is thus indicated LEIKOLA (1962) observed that swimming strongly inhibited alcohol absorption from the intestinal tract, but had no such effect when the alcohol had been administered intraperitoneally, probably because his samples were drawn 90 min after injection Anyhow, the small reduction in blood alcohol observable 45 min after injection could not nearly account for the great change in performance caused by the swimming This experiment clearly indicates that the ACTH stimulated release of adrenocortical hormones normally occurring in stress is not *involved in the reduction of alcohol intoxication* observed after swimming

Experiment 2 Effects of Adrenalin and Amphetamine

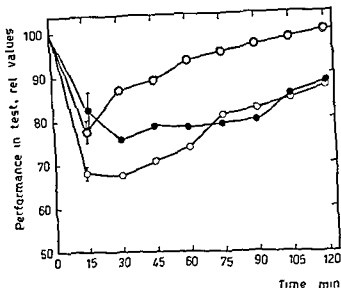
It was decided to test for a central arousal reaction, possibly mediated through medullary secretion of adrenaline, by injecting adrenaline or amphetamine in conjunction with alcohol Amphetamine mimics some of the peripheral effects of adrenaline on the autonomic nervous system, but it has a much stronger central action, being a powerful stimulant and acting especially on the brain-stem reticular activating system Within the central nervous system it may have "a direct arterenergic mode of action" (ROSSUM, VAN DER SCHOOT & HURKMANS 1962)

The experiments were performed on 20 male rats of the same age and partly from the same litters as those used in experiment 1 Because of this, the results of the control test done on the sham operated rats were used as reference Preliminary experiments led to the selection of 1 µg adrenaline and 5 µg amphetamine/g as suitable doses The rats were divided into two groups of ten (1 and 2) and were tested according to the schedule

a) group 1, 1 µg adrenaline/g (solution of 0 05 mg/ml in saline), group 2, 1 µg adrenaline and 2 mg alcohol/g (solution of 0 05 mg adrenaline and 100 mg alcohol/ml in saline),

b) three days later group 1, 5 µg amphetamine and 2 mg alcohol/g (solution of 0 25 mg amphetamine and 100 mg alcohol/ml in saline) group 2, 5 µg amphetamine/g (solution of 0 25 mg amphetamine/ml in saline)

Neither adrenaline nor amphetamine by itself had any influence on performance in the test The effect in conjunction with ethanol is shown in fig 2 Adrenaline improved the performance of the animals initially, the difference in effect 15 minutes after injection being statistically



significant (P < 0 025) Amphetamine had a significant effect throughout, bringing up the performance to the level induced by swimming stress at 75 minutes after injection There seemed to be some delay before the action of amphetamine became fully evident It should also be noted that amphetamine was without effect on the blood alcohol level, which at 45 min was higher in this experiment than after swimming (compare values for blood alcohol, expt 1)

The fact that both adrenaline and, more especially, amphetamine mimiced the effect of swimming was taken as supporting evidence for the notion that an arousal reaction was involved in the reduction of the apparent level of intoxication after swimming

Experiment 3 Effect of swimming and of Amphetamine on Performance after a Dose of 3 mg Alcohol/g

Leikola (1962) found that large doses of alcohol blocked the effect of swimming Experiment 3 aimed at an estimation of the dose required to reduce or block the stress effect, and to obtain similar information with respect to the effect of amphetamine

Twenty four rats obtained from Orion were divided into two groups, A and B, and first tested for possible differences after administration of

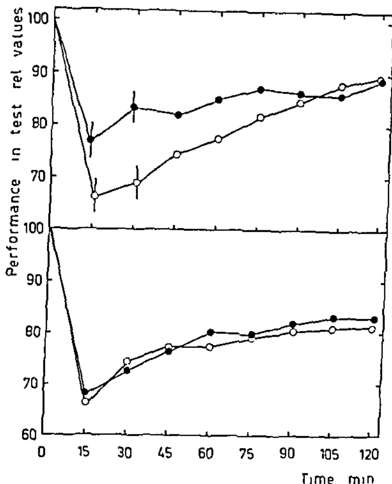


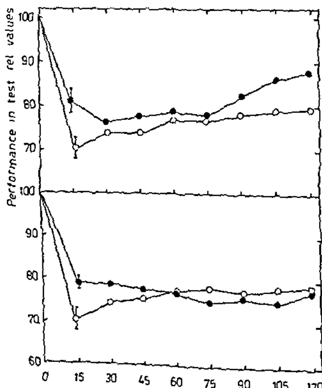
Fig 3 Performance in tilted plane test after administration of 3 mg alcohol/g averages for 12 rats in each gr
controls (O), alcoho ●

2 mg alcohol/g body weight The performance of the groups was identical Two days later, the effect of swimming was tested as in experiment 1 using A as control and B for the swimming test, and giving 3 mg of alcohol/g body weight After an interval of 3 days, group A was tested with a dose of 5 μg amphetamine and 3 mg alcohol/g body weight The results are shown in fig 3 There was still a clear response to swimming the effect on performance being statistically significant at both 15 and 30 minutes after injection ($P < 0.05$ and 0 005, respectively) Both the lowest values observed (65 ± 9 and 74 ± 8%), and the average performance (79 ± 7 and 85 ± 6) differed at the 5 per cent level However, the effect was much smaller and disappeared sooner than after a dose of 2 mg alcohol/g Amphetamine had no influence at all on the performance in the test after this larger dose of alcohol Since the result was clear, larger doses were not tried

This result may be interpreted as indicating that alcohol changes performance in the tilted plane test partly by influencing the activation level of the animals, and that this state of "partial sleep" is almost reversible after small doses of alcohol With larger doses, the reversibility of the effect of alcohol is reduced and even disappears

Experiment 4 Other Types of Stress

It seemed desirable to discover whether the reaction to swimming reflected a general effect of stressful conditions or was perhaps linked with the vigorous physical exercise involved Twenty four rats obtained from Orion and divided into two groups (I and II) were used in the experiment After a control test with 2 mg alcohol/g body weight, to exclude the



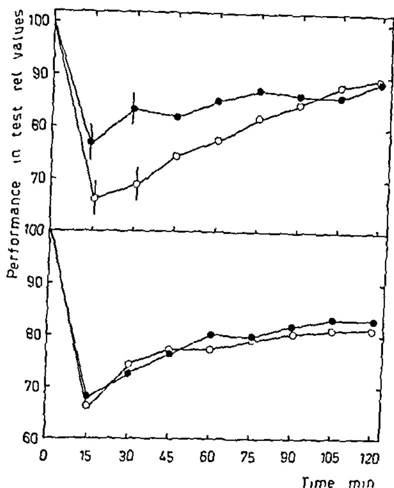


Fig. 3 Performance in tilted plane test after administration of 3 mg alcohol/g averages for 12 rats in each group Upper section controls (○) after swimming (●) Lower section controls (○) alcohol + 5 µg amphetamine/g (●) Vertical bars indicate standard error of the means for points found to differ significantly

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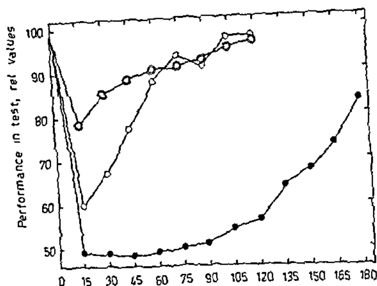


Fig 5 Performance in tilted plane test of immature, 11 week old female rats after administration of 2 mg (◎) 3 mg (O), and 4 mg (●) alcohol/g All groups consisted of 10 animals

reaction After 4 mg/g, the decrease in level of performance was as great as it would have been in older rats, but again rapid recovery was seen between 120 and 180 minutes

Comments and Conclusions

The persistence of the effect of swimming on intoxication led us to assume that some hormonal factor might underlie the phenomenon The improvement of performance was identical, however, in hypophysectomized and in control animals, in spite of the multiple endocrine and metabolic disturbances induced by removal of the hypophysis This fact suggests that a hormonally induced direct change of tolerance to alcohol at the neuronal level is not a major factor in the apparent change in level of intoxication after stress The observations with amphetamine and adrenaline, and with other types of stress, as well as the evident "threshold" in alcohol dosage above which stress does not influence performance, are evidence in favour of the view that an arousal reaction is involved The identical reaction of intoxicated hypophysectomized and unoperated animals to the swimming stress has a bearing on the physiology of arousal Besides strong neural stimulation, general humoral metabolic effects may be involved The much weaker effect of electric shock and of noise than of swimming is consistent with findings that

possibility of chance differences between the groups, they were tested twice at three-day intervals In the first test group I was exposed to electric shocks with group II as control; in the second testing, group II was subjected to a loud noise and group I was used as control The electric shocks were delivered from a cage floor consisting of brass rods, charged by means of a stimulator delivering a current of 500 μa and 1100 V, and provided with a scrambling device that changed the polarity of the rods Electric shocks were delivered for 5 min before and 5 min after injection of alcohol at a dose of 2 mg/g The animals were exposed to noise in cages measuring 18 5 × 18 5 × 16 cm and covered with sheet-iron Pieces of metal were shaken against the covers, the noise (ca 110 phone) continuing for 5 min before and after the injection of alcohol

The results of these experiments are shown in fig 4 The improvement in performance in the tilted plane test after these forms of stress was low At 15 min, there was a significant improvement in performance both after electric shocks ($P < 0\ 02$) and after exposure to noise ($P < 0\ 025$)

Experiment 5 Intoxication in Immature Rats

WALLGREN (1959) has reported a decrease in the alcohol tolerance of young rats between 14 and 22 weeks of age, and a marked sex difference at 14 weeks, the females being capable of a better performance in the tilted plane test than the males The dose employed was 2 mg alcohol/g body weight, administered intraperitoneally In view of the observations reported above, it appeared possible that the differences observed by WALLGREN (1959) were due to differences in the activation level of the animals, especially since young rats seem especially tense and fearful during handling compared with older ones Ordinarily, performance in the tilted plane test is linearly related to alcohol dosage (ARVOLA *et al*, 1958) If the assumed reversible action of alcohol on the activation system were involved in the apparent tolerance of the young rats, then a threshold effect of increasing alcohol dosage should appear as an abrupt decrease in the performance of the rats after a certain dose

Thirty female rats, 11-weeks old, were divided into three groups receiving 2 mg, 3 mg or 4 mg alcohol/g As shown in fig 5, the outcome of the experiment was in accordance with prediction With 4 mg/g, the effect up to 90 min after injection was too large to be measurable in the test, and hence no linear relationship to dosage could be shown Recovery after the first marked effect of 3 mg/g was extremely rapid (compare fig 3), probably reflecting "overshooting" of the alcohol level in the brain during the period of rapid absorption, and subsequent diminution of the brain alcohol level below the threshold value blocking the assumed arousal

tolerance appearing after administration of intoxicating doses of alcohol only a few times and without any concomitant changes indicated in the tolerance of the cerebral cortex It was suggested that subcortical centres might be involved, pointing to the possible involvement of activating mechanisms

Summary

Vigorous exercise radically improved the performance of rats in a simple functional test after 2 mg alcohol/g had been given intraperitoneally Other forms of stress – electric shocks and loud noise – had much less effect Hypophysectomized rats reacted as strongly as normal ones Thus activation of adrenocortical secretion by stress induced ACTH release is not involved in the reaction Results of experiments with adrenaline and amphetamine, the latter improving performance as much as the muscular work, suggest that an arousal reaction is involved The results are in agreement with the view that the brain stem reticular activating system is sensitive to alcohol, but that with moderate doses the effect is largely reversible The effect of exercise was diminished and that of amphetamine blocked after intraperitoneal injection of 3 mg alcohol/g, indicating that there is a threshold dose of alcohol above which the arousal reaction becomes difficult to achieve

Acknowledgements

We thank Dr Heikki Suomalainen, director of chemical research, for his interest in and compliance with the arrangements for the work reported here and Dr Olof Forsander for helpful criticism of the manuscript Miss Leena Saarinen, Mrs Kaija Salmela and Mr Kurt Åhman assisted in the experiments

REFERENCES

Arvola A L. Sammalisto & H [illegible]

Casp [illegible]

D Armour F E. & F D [illegible]
physiology 19 [illegible]

Friedman, S L. & [illegible]
for measuring [illegible]
alcohol intox[illegible]

Hogans A F O [illegible]
EEG and svc [illegible]
Physiol 1961, [illegible]

Jellinek E. M *The disease concept of alcoholism* 1960 Hillhouse Press, New Haven Conn

indicate the level of activation to depend among other factors on catechol amines (especially adrenaline and noradrenaline) and sympathetic nerve activity, both of which are raised during muscular activity (for a recent review of humoral effects on arousal, see SODERBERG 1962)

In connection with the physiology of alcohol intoxication, we find these observations of interest CASPERS (1958) has demonstrated electro physiologically a specific action of alcohol, shared by the barbiturates, on the brain stem reticular activating system In a recent review, KALANT (1962) has rightly placed great emphasis on the significance of CASPERS's observations for an understanding of the behavioural changes induced by alcohol Our impression is, however, that an especially noteworthy feature of this effect is its apparent lability in states of moderate intoxication Alcoholic intoxication is more inert than behavioural drowsiness, but it does not inhibit arousal, providing the stimulus, whether physiological or pharmacological, is strong enough

The tilted plane test appears to give an exaggerated view of the effect of stress and of amphetamine in counteracting the action of alcohol With both, motor incoordination was still visible in posture and movements The most obvious change was that the animals seemed much more alert and aware of the environment Normal exploratory behaviour, which largely disappears with the alcohol doses employed reappeared after swimming Amphetamine caused a state of general wakefulness and restlessness and strong "startle" responses to stimulation Since simple grasping holds a rat on the tilting plane the test is not a sensitive indicator of motor inco ordination Thus we would suggest that the tilted plane test largely measures effects on the activation level that will determine the response to tilting Although the influence of low doses of alcohol may be largely masked by such phenomena, the visible incoordination suggests that an effect on the cerebral cortex still persists

We consider it possible that the sex difference and the age dependent change in the effect of alcohol on the performance of rats in the tilted plane test, reported by WALLGREN (1959), are also due to differences in activation level, linked with the strong reaction to handling in young "frightened" animals

It remains to be shown whether changes in the resistance of the activating system to alcohol are involved in the well known behavioural tolerance of alcohol developed on continued use This question of increased "tissue tolerance ' has recently been reviewed by JELLINEK (1960) It is clear that tolerance is especially evident with moderate doses of alcohol According to JELLINEK, there are no reliable reports of changes in lethal doses HOGANS, MORENO & BRODIE (1961) using monkeys and WALLGREN & LINDBOHM (1961), using rats, have reported behavioural

tolerance appearing after administration of intoxicating doses of alcohol only a few times and without any concomitant changes indicated in the tolerance of the cerebral cortex It was suggested that subcortical centres might be involved, pointing to the possible involvement of activating mechanisms

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REFERENCES

[illegible]

Cas[illegible]

D Amour F E & F R Blood *Man[illegible]* *physiology* 1954 The Uni[illegible]

Friedman S L & J W Inga[illegible] for measuring the perform[illegible]

[illegible]

[illegible] L M The disease concept of alcoholism 1960 Hillhouse Press New Haven Conn

Kalant H Some recent physiological and biochemical investigations on alcohol and alcoholism A review *Quart J Stud Alc* 1962 **23**, 52 93
Leikola A Effect of stress on alcohol intoxication in rats *Quart J Stud Alc* 1962 **23**, 369 375
Rossum J M van J B van der Schoot & J A T Hurkmans Mechanism of action of cocaine and amphetamine in the brain *Experientia* 1962 **18** 229–231
Söderberg U The effect of blood borne influences on the cerebral cortex in wakefulness and sleep *Proc Int Un on Physiol Sciences Vol I XXII International Congress of Physiological Sciences Leiden* 1962 p 457
Wallgren H Sex difference in ethanol tolerance of rats *Nature* 1959 **184** 726–727
Wallgren H A Arvola & L Sammalisto The tilting plane test of alcohol intoxication in rats A response to the Friedman Ingalls critique *Quart J Stud Alc* 1960 **21** 698 700
Wallgren H & R Lindbohm Adaptation to ethanol in rats with special reference to brain tissue respiration *Biochem Pharmacol* 1961 **8** 423 424

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From the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa Canada

The Effect of Sodium Fluoride on the Actions of Succinylcholine, Parathion and Demeton in Rats.

By

W. B Rice and F. C. Lu

(Received November 24 1962)

The toxicity of fluoride has been extensively studied in domestic and laboratory animals, but its effects on the nervous system have received relatively little consideration. Lu *et al* (1961) reported that rats treated with sodium fluoride became more sensitive to convulsant agents, pentobarbital and diphenylhydantoin Cholinesterase inhibition was considered to be a possible mechanism of action, in an attempt to gain some information on this matter, the experiments to be described were done

Methods

Two series of experiments were done on female rats of a Wistar strain In the first series the rats were fed for 16 weeks on a diet of ground fox chow containing sodium fluoride in a quantity sufficient to provide 70 p p m of elemental fluoride

In the second series two groups of rats were given fluoride as sodium fluoride at 1 68 or 3 36 mg/100 g body weight This was given by stomach tube daily for 30 days At the end of the fluoride treatment periods, the determinations recorded below were made

Paralytic effect of succinylcholine The method described by Allmark & Bachinski (1949) was used to determine paralytic activity The drug was given by intramuscular injection, after which the rats were placed on a screen at an angle of 60 degrees Animals that slid off the screen within 20 minutes were taken as "positive", although most of the rats reacting to the drug fell within 7 minutes The PD50 was calculated by the probit method (Bliss 1935 a, b)

Toxicity of parathion and demeton The compounds were given in an aqueous suspension by intraperitoneal injection Ten rats were used at

Table 1

Effect of Fluoride Treatment on Succinylcholine and Parathion

Pre treatment	Succinylcholine PD50 ± SE (mg/kg)	Parathion LD50 ± SE (mg/kg)
Control – no fluoride treatment	0 7802 ± 0 0827	2 880 ± 0 272
Fluoride – 70 p p m in diet for 16 weeks	0 3789 ± 0 0690	3 106 ± 0 266

each dose level, and the numbers of animals dead within 48 hours were recorded The LD50 values were calculated by the probit method (BLISS 1935 a, b)

Results

In the first experiment, administering 70 p p m of fluoride in the food of rats did not change the toxicity of parathion compared to controls However, the paralytic activity of succinylcholine showed about a twofold increase These results are shown in table 1

In the second experiment, administration by stomach tube of 1 68 or 3 36 mg *fluoride per 100 g body* weight caused a moderate increase in toxicity of parathion and about a fivefold increase in that of demeton Pre treatment with fluoride at the lower dose was as effective as the higher dose in augmenting the toxicity of these two cholinesterase inhibitors as shown in table 2

Table 2

Effect of Fluoride Treatment on the Toxicity of Parathion and Demeton

Pre treatment	Parathion LD50 ± SE (mg/kg)	Demeton LD50 ± SE (mg/kg)
Control – no fluoride treatment	3 222 ± 0 177	3 202 ± 0 476
Fluoride 1 68 mg/100 gm body weight daily for 30 days	2 280 ± 0 099	0 664 ± 0 077
Fluoride – 3 36 mg/100 gm body weight daily for 30 days	2 240 ± 0 105	0 694 ± 0.083

Discussion

DYBING & LOE (1956) showed *that fluoride can inhibit in vitro* the cholinesterase systems of both plasma and erythrocytes of rats KOKETSU & GERARD (1956) reported that sodium fluoride augmented the end plate

potentials of the frog nerve sartorius preparation and increased the sensitivity of the end plate to applied acetylcholine

Succinylcholine is hydrolyzed *in vitro* by the pseudocholinesterase of plasma (GLICK 1941) and more slowly by the true cholinesterase of erythrocytes (BOVET NITTI 1949) GRAHAM *et al* (1957) showed an increase in toxicity of succinylcholine in rats pre treated with parathion, which is a cholinesterase inhibitor The enhanced paralytic effect of succinylcholine observed in the fluoride treated rats could be a result of inhibition of cholinesterase activity Moreover, since succinylcholine is cholinomimetic (CASTILLO & DE BEER 1950), sensitization of the motor end plate regions as suggested by KOKETSU & GERARD (1956) could also be a contributing factor

The toxicity of parathion was not affected in rats that had been given 70 p p m of fluoride in the diet, which corresponds to 0 84 mg per 100 g body weight In the rats receiving the two higher levels of fluoride there was a much smaller increase in the toxicity of parathion compared with that of demeton Parathion is converted in mammals to a more effective cholinesterase inhibiting analogue, paraoxon, through an oxidative enzyme s tem ... on consists ... which are ...) Thus the much smaller increase in toxicity of parathion could be due to inhibition by fluoride of the enzyme system that converts parathion to paraoxon, since fluoride is known to inhibit many enzyme systems (COLOWICK & CAPLAN 1955)

Parathion is eliminated through breakdown by a serum A esterase (ALDRIDGE 1953) Presumably demeton is handled in a similar manner Although no experimental evidence for it exists, it is reasonable to speculate that fluoride has an inhibiting effect on A-esterases Therefore it is suggested that the increase in toxicity of parathion and demeton in fluoride treated rats could be due to cholinesterase inhibition and possibly sensitization of motor end plates along with accumulation of parathion and demeton

Summary

Rats receiving fluoride at 70 parts per million in the diet for 16 weeks were more sensitive to the paralytic effects of succinylchol... conta...

... fluoride ... mg/100 g body weight by stomach tube daily for 30 days the toxicity of demeton was greatly increased, but that of parathion only slightly

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From the Department of Physiology, Karolinska Institutet
Stockholm 60, Sweden

Supersensitivity to Ethanol in Rabbits Treated with Coprinus atramentarius

By

R. Barkman and E. S. Perman[1])

(Received January 8, 1963)

It has long been known that ethanol, even in small amounts, elicits a series of characteristic unpleasant symptoms in persons who have eaten the mushroom *Coprinus atramentarius* Fries (CHIFFLOT 1916; FISCHER 1945 and others) The remarkable similarity between the manifestations of this supersensitivity to ethanol and those of the disulphiram-ethanol reaction (HALD, JACOBSEN & LARSEN 1948) led to speculation that disulphiram might exist in *C atramentarius* (JOSSERAND 1952), and SIMANDL & FRANC (1956) reported that they had isolated disulphiram from *C atramentarius* However, WIER & TYLER (1960) were unable to confirm this and concluded that the disulphiram like activity exhibited by *C atramentarius* was due to some other substance No further attempt to study the nature of this substance seems to have been reported – A dramatic description of an accidental *C atramentarius*-ethanol reaction in a cow has recently been given by CLÉMENÇON (1962)

It has recently been shown that a disulphiram ethanol reaction can be produced in rabbits, and that this reaction corresponds well with the human reaction in several important features (PERMAN 1962 a, b) Our experiments were carried out to establish whether or not the rabbit is also rendered supersensitive to ethanol by ingestion of *C atramentarius*

Methods

Naturally-occurring carpophores of *Coprinus atramentarius* Fries, supplied by a mycological expert, were carefully scraped clean and stored at –20°C for about 3 months Suspensions were prepared by homogenizing known amounts of the mushroom and suspending the homogenate in sucrose solution (63% w/w) containing 24% (w/v) gum arabic Similar suspensions were also prepared from the common

1) Recipient of a research grant from the Swedish State Medical Research Council

REFERENCES

Aldridge W N An enzyme hydrolyzing diethyl p nitrophenyl phosphate and its identity with the A esterase of mammalian sera *Biochem J* 1953 53 117 124

Allmark M G & W M Bachinski A method of assay for curare using rats *J Am Pharm Assoc Sci Ed* 1949 38 43–45

Bliss C I The calculation of the dosage mortality curve *Ann Appl Biol* 1935 22 134–167

Bliss C I The comparison of dosage mortality data *Ann Appl Biol* 1935 22 307 333

Bovet Nitti F Degredazione di alcune sostanze curarizzante per azione d colinesterasi *Rend ist s per sanita* 1949 12 138 157

Castillo J C & E J de Beer The neuromuscular blocking action of succinylcholine (diacetylcholine) *J Pharmacol Exptl Therap* 1950 99 458–464

Colowick S P & N O Kaplan (eds) *Methods in Enzymology* Vol II Academic Press New York 1955

Deichmann W B & R Racoczy Toxicity and mechanism of action of Systox *A M A Arch Ind Health* 1955 11 324–331

Dybing O & L V Loe Fluoride poisoning and cholinesterase in rats *Acta pharmacol et toxicol* 1956 12 364 368

Glick D Some additional observations on the specificity of cholinesterase *J Biol Chem* 1941 137 357 362

Graham R C B F C Lu & M G Allmark The effect of some organo phosphorus and chlorinated hydrocarbon insecticides on the toxicity of several muscle relaxants *J Pharm Pharmacol* 1957 9 312 319

Holtz P & E Westermann *Giftung und Entgiftung von Parathion und Paraoxon* *Naunyn Schmiedebergs Arch Exp Path Pharmak* 1959 237 211 21

Koketsu K & R W Gerard Effects of sodium fluoride on nervemuscle [illegible] 1956 186 278 282

Lu [illegible] Allmark & P Boivin [illegible] various central nervous [illegible] 1961 3 31 38

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Methods

Naturally-occurring carpophores of *Coprinus atramentarius* Fries supplied by a mycological expert were carefully scraped clean and stored at −20 C for about 3 months Suspensions were prepared by homogenizing known amounts of the mushroom and suspending the homogenate in sucrose solution (63% w/w) containing 24% (w/v) gum arabic Similar suspensions were also prepared from the common

1) Recipient of a research grant from the Swedish State Medical Research Council

REFERENCES

Aldridge W N An enzyme hydrolyzing diethyl p nitrophenyl phosphate and its identity with the A esterase of mammalian sera *B ocl em J* 1953 53 117 124

Allm . ' '' r r

Bliss (, · ·
1935 22 134–167

Bliss C I The comparison of dosage mortality data *Ann Appl B ol* 1935 22 307 333

Bovet Nitti F Degredazione di alcune sostanze curarizzante per azione d colinesterasi *Rend Ist s iper sanita* 1949 12 138 157

Castillo J C & E J de Beer The neuromuscular blocking action of succi nylcholine (diacetylcholine) *J Pharmacol Exptl Therap* 1950 99 458–464

Colowick S P & N O Kaplan (eds) *Methods in En yn ology* Vol II Academic Press New York *1955*

Deichmann W B & R Racoczy Toxicity and mechan sm of action of Systox *A M A Arch Ind Health 1955* 11 *324 331*

Dybing O & L V Loe Fluoride poisoning and cholinesterase in rats *Acta pharmacol et toxicol* 1956 12 364 368

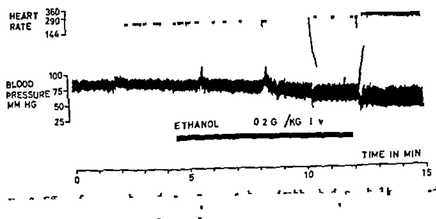
Glick D Some additional observations on the specificity of chol nesterase *J Biol Chem* 1941 137 357 362

Graham R C B F C Lu & M G Allmark The effect of some organo phosphorus and chlorinated hydrocarbon insecticides on the toxicity of several muscle relaxants *J Pharm Pharmacol* 1957 9 312 319

Holtz P & E Westermann Giftung und Entg ftung von Parathion und Paraoxon *Naunyn Schmiedebergs Arch Exp Path Pharmak* 1959 237 211–21

Koketsu K & R W Gerard Effects of sodium fluoride on nervemuscle transmission *Am J Phys ol* 1956 186 278 282

Lu F C I M Mazurkiewicz R S Grewal M G Allmark & P Boivin The effect of sodium fluoride on responses to various central nervous system agents in rats *Toxicol Appl Pharmacol* 1961 3 31 38



rate recordings from a rabbit that reacted strongly to as little as 0 2 g/kg ethanol This animal died in a state of severe hypotension after the subsequent dose of 0 4 g/kg had been infused

No corresponding reaction after ethanol administration was seen in rabbits treated with 20 g of *C atramentarius*, 50 g of *Psalliota hortensis* or in untreated animals

Discussion

The results recorded show that the rabbit is rendered supersensitive to ethanol by moderate amounts of *C atramentarius* The rabbit is also rendered supersensitive to ethanol by disulphiram (PERMAN 1962 b) and calcium carbimide (temposil ®, Lederle) (PERMAN, unpublished) All these agents are well known to induce supersensitivity to ethanol in man This gives further support to the assumption that supersensitivity to ethanol is elicited by similar mechanisms in both species

Use of the rabbit as test animal should facilitate attempts to isolate and define the chemical structure of the active agent in *C atramentarius* The possibility of a therapeutic application of this agent as an alternative adjunct in the treatment of alcoholism appears to be a major reason for undertaking such work – Preliminary results indicate that the active agent can be extracted with ethanol (BARKMAN & PERMAN, unpublished)

Summary

Small ethanol doses (0 2–0 6 g/kg i v) regularly produce long lasting hypotension and hyperventilation in unaesthetised rabbits receiving

mushroom *Psalliota hortensis* The suspensions were administered by stomach tube within one hour of preparation No animal received more than 50 ml of the suspension

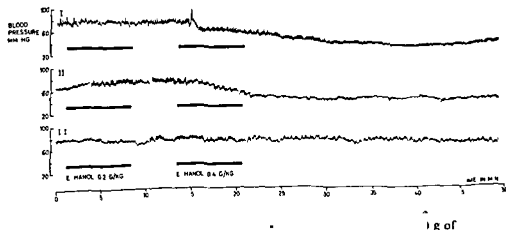
In male rabbits (2 5–3 5 kg) polyethylene catheters were inserted into the central artery and marginal vein of one ear under local anaesthesia as described by PERMAN (1962 b), a technique permitting repeated blood pressure recordings in the same animal these were made with an ink-writer (Varian Model G 10 Recorder, Varian Ass , U S A) via a strain gauge transducer (Elema, Sweden)

Ethanol was infused i v diluted in saline (0 9% v/v) at rate of 1 0 ml/min 4 hours after pretreatment As a rule 0 2 g/kg was given first and then 0 4 g/kg after an interval of 5 min The blood pressure was recorded for at least 30 min after the end of the latter dose Results of experiments on 3 rabbits treated with a suspension containing 50 g of *C atramentarius* (corresponding approximately to 5 mushrooms), 2 rabbits treated with an extract containing 20 g of *C atramentarius*, 2 rabbits treated with an extract containing 50 g of *Psalliota hortensis* and 3 untreated rabbits are reported

Results

The general condition of the rabbits treated with mushroom suspensions was not appreciably affected, judging from their appearance and behaviour The initial blood pressures were in the same range as those of untreated animals (70–100 mm Hg)

Rabbits treated with 50 g of *C atramentarius* regularly responded in 30 min or less of ethanol administration (0 2–0 6 g/kg) with a clear cut fall in blood pressure to 50–60 mm Hg, a moderate tachycardia and an easily noticeable hyperventilation Fig 1 shows that a blood pressure response to ethanol was obtained more than 24 hours after administration of the *C atramentarius* suspension Fig 2 shows blood pressure and heart



) g of

III Seventy two hours after pretrea

Acta pharmacol et toxicol 1963 20, 47–55

From the Department of Pharmacology University of Goteborg,
Goteborg Sweden

On the Role of the Liver Catechol O-Methyl Transferase in the Metabolism of Circulating Catecholamines[1])

By

Arvid Carlsson and Bertil Waldeck

(Received January 16 1963)

The main metabolic pathway for circulating catecholamines appears to involve formation of O methylated derivatives (AXELROD *et al* 1958, CROUT *et al* 1961, DE SCHAEPDRYVER & KIRSHNER 1961) LUND (1951) found that the liver has a high capacity for inactivating noradrenaline (NA) and later AXELROD & TOMCHICK (1958) demonstrated the high catechol O-methyl transferase (COMT) activity of this organ After intraperitoneal administration most of a drug will pass through the portal vein after absorption These facts prompted us to compare the tissue uptake of intravenously and intraperitoneally injected NA both in the presence and in the absence of a COMT inhibitor

Materials and Methods

Mice weighing about 20 g were divided into groups of six Each group received 10 µg/kg (in one experiment 2 µg/kg table 4) ^{3}H NA[2]) intraperitoneally and then immediately 10 µg/kg ^{14}C-NA[3]) intravenously The tissues to be analyzed were removed from the beheaded animals pooled and extracted with perchloric acid as described by BERTLER CARLSSON & ROSENGREN (1958) Ten minutes after the injection the activity remaining in the peritoneal cavity was found to be less than 10% of that of the above dose of NA given in 0 5 ml isotonic saline solution

After add ng 2 mg ascorb c acid and 20 [illegible]

r ne
m de
tetrac

[2]) [illegible] 1 55 C/mM was obta ned from New England Nuclear Corporat on Boston U S A

[3]) Noradrenal ne-β-^{14}C acetate 21 9 mC/mM was obtained from Comm ssariat a l'Energie Atom que G f sur Yvette, France

orally suspensions of the mushroom *Coprinus atramentarius* Fries Such supersensitivity to ethanol persisted for more than 24 hours in an animal receiving about 50 g of *C. atramentarius*. No hypotension or hyperventilation occurred when ethanol was administered to untreated rabbits or rabbits treated with a corresponding amount of the mushroom *Psalliota hortensis*. The similarily between the *C. atramentarius*-ethanol reaction and the disulphiram-ethanol reaction in the rabbit is pointed out – Use of the rabbit as test animal should facilitate attempts to isolate and determine the chemical structure of the agent in *C. atramentarius* that induces supersensitivity to ethanol.

Acknowledgement

The skillful technical assistance of Miss. A.-M. Hjort is gratefully acknowledged.

REFERENCES

Chifflot, M J : Sur un cas de rubéfaction de la face, tendant à se généraliser à la suite de l'ingestion du *Coprinus atramentarius* Fr *Bull Soc Mycol France* 1916, 32, 63

Clémençon, H : Antabus-Wirkung bei Kuhen, *Schweiz Z Pilzkunde* 1962, 40, 170–172

Fischer, I : Säregen svampförgiftning *Svenska Läk.-Tidn* 1945, 42, 2513–2515.

Goldschmidt, H and P Lindgren An electronic interval recorder for measuring peripheral blood flow and heart rate *J appl Physiol* 1962, 17, 169–171.

[illegible] effect of tetraethylthiu- [illegible] *et toxicol* [illegible] yl alcohol [illegible] 1962 a, 18, 516

Perman, E S Studies on the Antabuse-alcohol reaction in rabbits *Acta physiol scand* 1962 b, 55, Suppl 190

Simandl, J and J Franc Isolace tetraethylthiuramdisulfidu z hniku inkoustového *Chem listy* 1956, 50, 1862–1863

Wier, J. K and V. E Tyler An investigation of *Coprinus atramentarius* for the presence of disulfiram *J Amer pharm Ass, sci Ed* 1960, 49, 426–429

Table 1.

Recovery of Noradrenaline

Tissue	% recovery from homogenates	% recovery from extracts
Heart . .	80	97
	78	92
Femoral muscle .	81	99
	81	96

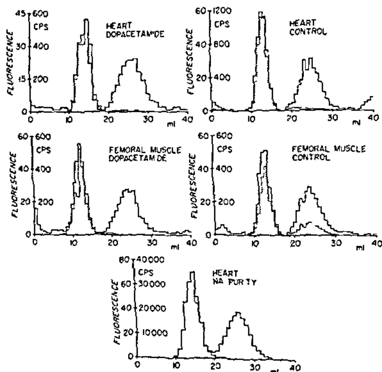
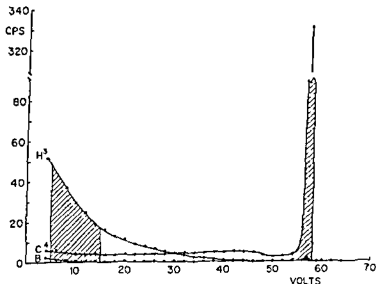


Fig. 2. Ion exchange chromatography of tissue extracts of heart and femoral muscle. The animals received ^3H NA intraveno[illegible]

diam 4 mm, height at pH 0 90 mm), buffered with 25 ml 0 1 N phosphate buffer at pH 6 5 The column was washed with 40 ml water, and elution was performed by N hydrochloric acid in three steps The first 8 ml were discarded The next 9 ml (fraction 1) contained the NA and the third 15 ml (fraction 2) the NM (HAGGENDAL 1962) The eluates were evaporated to dryness under reduced pressure in a rotating evaporator at 35°C, and the residues were each taken up in 1 ml 99 5% ethanol containing 1% concentrated hydrochloric acid After adding 5 ml scintillation mixture[1]) 5 ml of the sample were transferred to a counting vial and analysed for their content of ^3H and ^{14}C in an EKCO liquid scintillation counter[2]) by means of pulse height analysis Fig 1 shows pulse height spectra for both isotopes and for the background In order to raise the ^3H pulses over the discriminator threshold and thus to obtain increased counting efficiency, the preamplifier was overloaded, which is the reason for the distortion of the ^{14}C spectrum The left shaded area represents the counting range for ^3H and the right one that for ^{14}C The interference of ^3H in the ^{14}C range can be neglected, but a correction must be made for interference of ^{14}C in the ^3H range as follows

Let S^3H be the true counting rate for ^3H in the sample, S1 the counting rate for the sample at the ^3H range, S2 the counting rate for the sample at the ^{14}C range, C1 the counting rate for the ^{14}C standard at the ^3H range, and C2 the counting rate for the ^{14}C standard at the ^{14}C range

thus $S^3H = S1 - \frac{C1}{C2} S2$



[illegible] obtained against

[1]) 3 g 2 5 Diphenyloxazol (PPO) and 0 3 g 1 4 Di [2 (5 phenyloxazolyl)] benzene (POPOP) in one liter toluene
[2]) N 664A + scaler N 610A

Table 1.

Recovery of Noradrenaline

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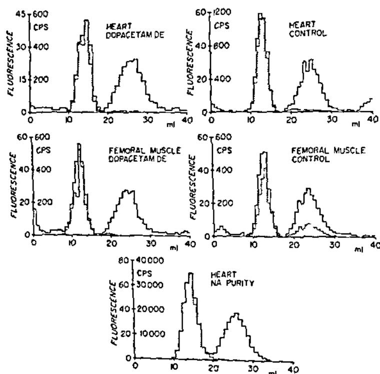


Fig 2 Ion exchange chromatography of tissue extracts of heart and femoral muscle The animals received ^{3}H-NA intravenously and were [illegible]
was given intrap[illegible]
adding labelled a[illegible]

diam 4 mm, height at pH 0 90 mm), buffered with 25 ml 0 1 N phosphate buffer at pH 6 5 The column was washed with 40 ml water, and elution was performed by N hydrochloric acid in three steps The first 8 ml were discarded The next 9 ml (fraction 1) contained the NA and the third 15 ml (fraction 2) the NM (HAGGENDAL 1962) The eluates were evaporated to dryness under reduced pressure in a rotating evaporator at 35°C, and the residues were each taken up in 1 ml 99 5% ethanol containing 1% concentrated hydrochloric acid After adding 5 ml scintillation mixture[1]) 5 ml of the sample were transferred to a counting vial and analysed for their content of ^{3}H and ^{14}C in an EKCO liquid scintillation counter[2]) by means of pulse height analysis Fig 1 shows pulse height spectra for both isotopes and for the background In order to raise the ^{3}H pulses over the discriminator threshold and thus to obtain increased counting efficiency, the preamplifier was overloaded, which is the reason for the distortion of the ^{14}C spectrum The left shaded area represents the counting range for ^{3}H and the right one that for ^{14}C The interference of ^{3}H in the ^{14}C range can be neglected, but a correction must be made for interference of ^{14}C in the ^{3}H range as follows

Let $S^{3}H$ be the true counting rate for ^{3}H in the sample, S1 the counting rate for the sample at the ^{3}H range, S2 the counting rate for the sample at the ^{14}C range, C1 the counting rate for the ^{14}C standard at the ^{3}H range, and C2 the counting rate for the ^{14}C standard at the ^{14}C range

thus $S^{3}H = S1 - \frac{C1}{C2} S2$

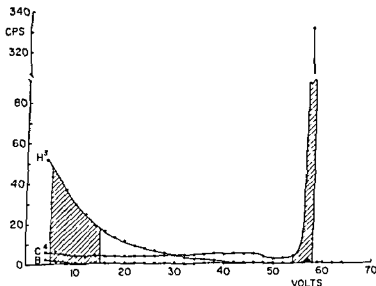


Fig 1 Pulse height spectra for tritium (^{3}H) carbon 14 (^{14}C) and background (B) obtained from an EKCO liquid scintillation counter Figure shows the counting rate plotted against discriminator threshold voltage

Instrument settings Gate width 1 volt
High voltage 1100 volts
Pre amplification 500

1) 3 g 2 5 Diphenyloxazol (PPO) and 0 3 g 1 4 Di [2 (5 phenyloxazolyl)] benzene (POPOP) in one liter toluene

2) N 664A + scaler N 610A

of untreated animals The radioactivity of the ordinary fraction 2 as defined above, from heart tissue does not seem to be derived from NM only, since a significant part of the activity left the column earlier than NM Prepreatment with the COMT inhibitor dopacetamide seemed to unmask this unidentified part of fraction 2, as shown in fig 2

In one experiment ^{3}H NA was added to a heart homogenate (fig 2, bottom) No impurity or artificial conversion product related to the unidentified activity in fraction 2 could be detected The results therefore suggest that the unidentified material had been formed *in vivo*

All values corresponding to fraction 2 were calculated on the assumption that the molecular weight was that of NM

[illegible]

used in the experiments were from time to time checked by radiopaper chromatography

In this paper radioactivity results for the NM fraction will be shown for skeletal muscle only From heart this fraction was not considered to be sufficiently pure to justify presentation of results

The results for radioactive NA and NM in tissues have been expressed as ng/g tissue (One ng = 10^{-9} g)

Results

The uptake of intraperitoneally administered NA by heart and skeletal muscle was about 30% of that injected by the intravenous route The radioactive content of NA was much higher in heart than in skeletal muscle and disappeared more rapidly from the latter (table 2)

Attempts were made to block the COMT Animals were pretreated with dopacetamide, a COMT inhibitor of low toxicity (CARLSSON *et al* 1962), 1 g/kg intraperitoneally 30 min before the administration of NA, and were killed 30 min thereafter The COMT inhibitor caused an increased uptake by heart and skeletal muscle of intravenously and particularly of

Table 2

Uptake of NA after injection of labelled NA Results in ng/g tissue ± standard error of the mean Figures in brackets indicate number of observations in each group

Tissue	Time hours	NA intraperitoneally		NA intravenously	
		NA	NM	NA	NM
Heart	0.5	3.69 ± 0.21 (7)	–	15.87 ± 2.06 (7)	–
	1	4.33 ± 0.36 (7)	–	19.25 ± 2.48 (6)	–
	2	3.32 ± 0.25 (4)	–	12.41 ± 2.29 (4)	–
Fem muscle	0.5	0.27 ± 0.02 (8)	0.30 ± 0.03 (8)	0.81 ± 0.03 (8)	0.45 ± 0.02 (8)
	1	0.19 ± 0.01 (7)	0.13 ± 0.03 (7)	0.69 ± 0.12 (7)	0.37 ± 0.14 (5)
	2	0.12 ± 0.02 (4)	0.05 ± 0.01 (4)	0.40 ± 0.14 (4)	0.08 ± 0.02 (4)

Recovery. The recovery throughout the procedure was estimated as follows. Heart and femoral muscle from twelve mice were homogenized in perchloric acid. ^{3}H-NA was added to the homogenates and after centrifugation ^{14}C-NA was added to the extracts. The extracts were divided into two parts, and each part was taken through the whole procedure. Recoveries from homogenates were about 80%, from extracts over 90% (table 1). Recoveries of inactive NM from extracts were over 90%. No corrections for incomplete recovery have been made.

Specificity. In some experiments on mice that had received radioactive NA, 10 µg NA and 10 µg NM were added to the tissue extracts as carriers. Then the extracts were treated in the usual way, except that the elution was performed on fractions of about one ml. Fluorescence and radioactivity were measured in each fraction (fig. 2). The first peak of radioactivity was always found to coincide with the fluorescence of the carrier NA. The radioactivity of the subsequent fraction, however, showed good agreement with the fluorescence of carrier NM only in that from the femoral muscle

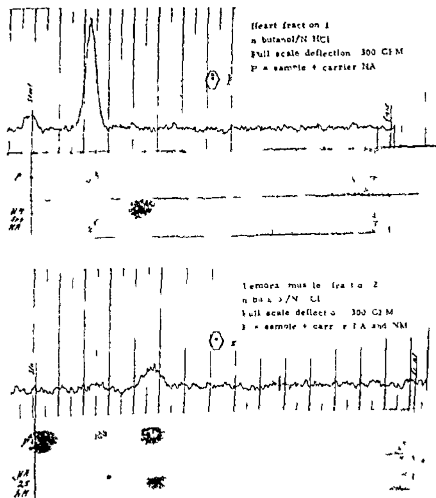


Fig. 3. Radio paper chromatography of fraction 1 of heart and fraction 2 of femoral muscle.

Table 4

[illegible] controls treated identically, but no [illegible]

Tissue	Hours	NA intraperitoneally		NA intravenously
		NA	NM	NA
Heart treated mice	0 5	2 8	-	32
	1 0	2.9	-	37
	2 0	2 5	-	27
Heart control mice	0 5	1 08	-	29
	1 0	0 93	-	23
	2 0	0 68	-	18
Femoral muscle, treated mice	0 5	0 19	0 013	1 78
	1 0	0 13	0 024	1 55
	2 0	0 11	0 022	0 90
Femoral muscle control mice	0 5	0 074	0 113	1 26
	1 0	0 065	0 057	1 11
	2 0	0 037	0 031	0 69

In general, the radiactive NA increased with increasing dosage in both the tissues examined

The use of the more potent α propyl derivative of dopacetamide (CARLSSON *et al* 1963) gave similar results, except that a dose of 0 5 g/kg intraperitoneally was then sufficient to cause marked inhibition of the enzyme (table 4)

Discussion

The lower recovery of labelled NA from tissues after intraperitoneal compared with intravenous injection is probably due to destruction during the passage through the liver The results suggest that COMT is involved in this destruction Pretreatment of the animal with a COMT inhibitor caused a pronounced increase in the amount of labelled NA recovered from tissues after intraperitoneal injection At the same time a decrease in tissue NM was observed After intravenous injection the effect of the COMT inhibitor, though present, was less marked This smaller effect seems to be of the same order as has been previously reported to occur after pyrogallol administration (AXELROD 1960) A considerable part – some 30% – of the cardiac output passes through the liver, so that this effect may be largely due to inhibition of liver COMT

Skeletal muscle took up much less NA than did heart tissue This is

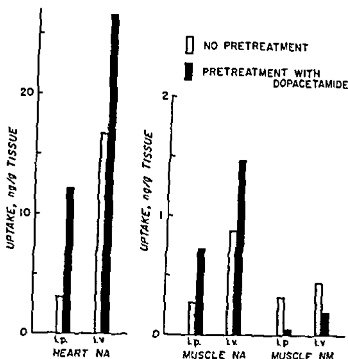


Fig 4 Effects of dopacetamide on tissue uptake of labelled NA
The drug was given intraperitoneally at a dose of 1 g/kg After 30 min labelled NA was given intraperitoneally (^{3}H-NA) and intravenously (^{14}C-NA) The animals were killed 30 min later

Table 3

Effects of three doses of dopacetamide on tissue uptake of NA Drug given 30 min before labelled NA and animals killed 60 min later Uptake of labelled NA in ng/g tissue

Pretreatment		Heart		Femoral muscle	
		NA intraperitoneally	NA intravenously	NA intraperitoneally	NA intravenously
Intra-peritoneally	No	5 6	18 0	0 26	0 74
	0 5 g/kg	10 1	23 8	0 60	1 12
	1 0 g/kg	12 3	22 7	0 64	1 14
	2 0 g/kg	20 0	55 0	1 07	4 07
Intra-venously	0 5 g/kg	8 8	21 1	0 46	1 03
	1 0 g/kg	5 8	25 0	0 43	1 58
	2 0 g/kg	9 3	36 0	0 65	2 15

intraperitoneally administered NA, whereas the radioactivity of the NM fraction was lowered (fig. 4 and fig 2).

In another experiment we compared the effects of three doses of the drug, 0 5, 1.0, and 2 0 g/kg body weight. The animals were pretreated 30 min. before and killed 60 min. after the administration of NA (table 3)

Axelrod J The fate of adrenaline and noradrenaline In J R Vane, G E Wolstenholme and Maeve O Connor (Editors) *A Ciba Foundation Symposium on Adrenergic Mechanisms* London 1960 28 39

Bertler, Å, A Carlsson & E. Rosengren A method for the fluorimetric determination of adrenaline and noradrenaline in tissues *Acta physiol scand* 1958 44 273 292

Carlsson A, M Lindqvist S Fila Hromadko & H Corrodi Synthese von Catechol O methyl transferase hemmenden Verbindungen In den Catecholaminmetabolismus eingreifende Substanzen 1 Mitteilung *Helv chim acta* 1962 45 270–276

Carlsson A., M Lindqvist, S Fila Hromadko & H Corrodi Synthese von Catechol O methyl transferase hemmenden Verbindungen In den Catecholaminmetabolismus eingreifende Substanzen 2 Mitteilung *Helv* [illegible]

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131 433–449

Hertting G J Axelrod, I J Kopin & L. G Whitby Lack of uptake of catecholamines after chronic denervation of sympathetic nerves *Nature* 1961, 189 66

Häggendal J On the use of strong exchange resins for determinations of small amounts of catecholamines *Scand J clin Lab Invest* 1962 14 537 544

Kopin, I J, G Hertting & E. K. Gordon Fate of norepinephrine-³H in the isolated perfused rat heart *J Pharmacol exp Ther* 1962 138 34–40

Lund A Elimination of adrenaline and noradrenaline from the organism *Acta pharm tox* 1951 7, 297–308

Whitby L. G, J Axelrod & H Weil Malherbe The fate of ³H Norepinephrine in animals *J Pharmacol exp Ther* 1961, 132 193 201

probably due to its much smaller supply of adrenergic nerves, which appear to be mainly responsible for the capacity of tissues to bind NA (HERTTING *et al.* 1961). The lower binding capacity of skeletal muscle is also reflected in a more rapid disappearance of labelled NA. The radioactive NM disappeared rapidly also. The results support the conclusion of WHITBY *et al.* (1961) that binding is an important factor in the inactivation of circulating NA. In heart it is probably the dominating factor, as suggested by the results of KOPIN *et al.* (1962), and this may be true also for other tissues with an abundant supply of adrenergic nerves.

Although extrahepatic COMT does not seem to play a major role in the inactivation of circulating catecholamines, further investigation is necessary for elucidation of its possible importance for the proper functioning of the adrenergic transmission mechanism.

Summary

Mice were injected with ^{3}H-noradrenaline (NA) intraperitoneally and ^{14}C-NA intravenously. The uptake of labelled compounds in heart and skeletal muscle and their conversion to normetanephrine (NM) were studied. The fraction of intraperitoneally administered NA recovered from the tissues was about 30% of the corresponding fraction of intravenously administered NA, indicating that most of the NA passing through the liver was destroyed. After treatment with the catechol O methyl transferase (COMT) inhibitor dopacetamide or its α-propyl derivative, the amount of NA derived from the intraperitoneal injection recovered from the tissues increased several fold, whereas the NA derived from the intravenous injection showed only a moderate increase. Enzyme inhibitors caused a considerable decrease in labelled NM.

In mice the liver COMT appears to play a dominating role in the metabolic conversion of circulating NA.

Acknowledgements

This work was supported by the Air Force Office of Scientific Research of the Air Research and Development Command, United States Air Force.

For technical assistance we thank Miss Inger Alexandersson and Miss Gun Holmberg.

REFERENCES

other catecnols

stance sulphacetamide sodium (mol wt 254 25) was employed and 100 mg/kg was injected subcutaneously into a rabbit as a 5% solution (SCHOU 1958) Eight rabbits were submitted to cross-over experiments In half the animals the control experiment was done first the other half undergoing the test with Ubiquin before the control experiment A period of at least 8 days elapsed between the two experiments on each animal

subcutaneous injection of sulphacetamide sodium Blood was sampled from the opposite ear marginal vein which was punctured by means of a phlebotome

A quantity of 250 µl blood and the same volume of heparin solution were blown

in table 1

Spreading Experiments

The method described by SCHMIDT (1956) was employed in the spreading experiments By this technique 0 1 ml test solution is injected subfascially on the inner side of the skin of mice which are killed by a blow on the head immediately before the spreading experiments The test solutions used were 1 Saline, 0 9%, 2 5 ml 0 5% solution of ubiquin added to 45 ml saline, 3 0 5 ml 0 5% ubiquin added to 49 5 ml saline (0 9%)

At 30 minutes after the injections (two on each mice) the diameters of the vesicles were measured and entered into the formula for the area of an elipse ($A = d_1 \cdot d_2 \cdot \frac{\pi}{2}$). All measurements were taken by a person unaware of the composition of the fluid in the vesicles

Capillary Permeability

Unanaesthetised female guinea pigs weighing 350–380 g were fixed in the ventral position on a special table The fur on the back was
depilator (wheat flour g 35, talcum
to form the consistence of porric

made isotonic by add

wh
by
0 0
sol
mine diphosphate 50 µg ml, 9 5 hydroxytryptamine as its creatinine sulphate (Sigma),

Acta pharmacol. et toxicol 1963, 20, 56–64

From the Department of Pharmacology University of Copenhagen
(Professor Knud O Møller, M D)

Investigations into the Properties of a Substance claimed to increase Absorption (Ubiquin ®)

By

Jens Schou

(Received January 18, 1963)

It has recently been reported that a synthetic substance named ubiquin ® acts as a spreading factor in connective tissue It was further described as able to enhance the rate of absorption of chemical substances from solutions injected subcutaneously when given locally together with them (PABST 1961) The substance is a polymer of oligo-N-methyl morpholonium-propylene-oxide-chloride, having a mean molecular weight of 1650

Several chemical substances are spreading factors in connective tissue-, for example, enediols containing a vicinal carbonyl group (DAUBENMERKL 1953) However, only hyaluronidase (and histamine antagonists) are known to increase the rate of absorption when given with substances injected subcutaneously (SCHOU 1961) It was suggested to us that the intravenous injection of ubiquin might possibly lead to an increase in the absorption rate of substances injected simultaneously by the subcutaneous route, if so this substance would possess pharmacological characteristics never described before It was therefore submitted to further pharmacological evaluation However, with the sample at our disposal we were unable to show any enhancing effect of intravenously injected ubiquin on the absorption rate of subcutaneously injected test substances Further, our results did not reveal characteristics that would be expected in a substance promoting absorption

Experiments and Methods

Absorption Experiments

Young adult male rabbits weighing 2 0–2 5 kilos, had free access to food under constant environmental conditions until the beginning of the experimental period During the absorption experiment lasting one hour the unanaesthetised animals were suspended in the dorsal position on a padded and heated rabbit table As test sub

Table 1

[illegible] ments on 8 rabbits [illegible]

Minutes after injection	Blood concentration (µg/ml)	
	Control (n = 8)	Ubiquin (n = 8)
5	20 1	21 7
10	38 2	39 0
15	51 9	52 3
20	59 5	61 9
25	64 8	66 5
30	66 4	68 9
45	64 8	66 3
60	60 8	59 4

Table 2

Spreading experiments on recently killed mice having 0 1 ml test solution injected subfascially into the underside of the skin Two diameters of the vesicles were measured after 30 minutes and the areas were calculated No significant differences existed between the figures from the experimental groups (t test, $P > 0\ 05$)

	Area in mm²	Number of experiments
Saline	79 4 ± 2 5	40
Ubiquin 0 5 mg/ml	77 3 ± 2 7	37
Ubiquin 0 05 mg/ml	75 2 ± 2 4	37

produced by the addition of ubiquin to the test solutions up to a concentration of 0 5 mg/ml, equivalent to about 0 3 mM

The results of the experiments on the *capillary permeability* of guinea pigs are summarized in fig 1 Ubiquin was found to be a "capillary permeability factor", showing a log dose response relationship almost exactly parallel to those for histamine and 5 hydroxytryptamine (shown in the same figure) To the test solution containing the highest concentration of ubiquin were added 5% glucose In control experiments it was found that glucose itself at this isotonic concentration provokes the development of a blue colour In 24 experiments the mean diameter of this reaction amounted to 3 8 mm However, 0 5% glucose in saline did not show any significant effect (24 experiments)

The readings given in response to the highest concentrations of ubiquin may in part have been provoked by the glucose The log dose response

50 µg/ml, 10 5-hydroxytryptamine creatine sulphate, 500 µg/ml Readings of the diameters of the blue areas were taken 30 minutes after the injections The experimental solutions were randomized and unknown to the person who took the readings

Isolated Guinea Pig Intestine

An isolated cleaned segment of guinea pig ileum was suspended in aerated (carboxygen Ph D, 96% v/v O_2 + 4% CO_2) Tyrodes solution at 37°. The sensitivity of the preparation was checked by adding known amounts of histamine hydrochloride Ubiquin was tested for histamine-like activity and histamine antagonism

Viscosimetry

Four viscosimeters of the type described by DALGAARD-MIKKELSEN & KVORNING (1948) were used. The flow-times for water held in a water bath at 37° ranged from 25 to 92 seconds

Potassium hyaluronate prepared from umbilical cords (JENSEN 1952) was dissolved in phosphate buffer, pH 6 8 The relative viscosity was adjusted by dilution to about 2 5–5 times the volume of the buffer solution The volume of the test solution in the viscosimeters was 250 or 500 µl After the constancy of viscosity had been checked for 30 minutes 50 µl 5% glucose in water, with or without 0 5% ubiquin, were added The flow time was then measured every second minute until it was constant.

Blood Pressure and Respiration

Rabbits and guinea pigs were anaesthetised with intraperitoneal urethane in 25% solution, so as to give 1 2 and 1 0 g/kg, respectively Rats were given intraperitoneal chlorbutol as described in Ph Nord vol IV p 89

For measurement of arterial blood pressure, a cannula was inserted into the carotid artery and connected to a presure transducer (Statham Instr Inc, P23AA, 0–750 mm Hg) by a water-filled length of polyethylene tubing A tracheal cannula was connected to a Fleisch tube (FLEISCH 1956), in which the rate of air flow is converted into a proportional pressure This pressure is measured by means of a Statham "static" low pressure transducer (sensitivity ± 7 mm H_2O)

The electrical system of each transducer was completed through amplifiers (Simonsen & Weel standard amplifier, type A 59) and an ink recorder (Elema Jet recorder, type TLS-8) By using two amplifiers and a special circuit the air speed is integrated so as to give the volume of air passing through the tube Calibration was obtained by 1 or 2 ml syringes The blood pressure records was calibrated by means of a column of mercury.

Results

Table 1 shows the results from 8 double cross-over *absorption experiments* on the subcutaneous tissue of rabbits. The rate of absorption was not significantly altered by the simultaneous intravenous injection of ubiquin.

The results from *spreading-experiment* on recently killed mice are given in table 2. No significant alterations in the size of spreading papules were

Table 3

Percentage decrease in relative viscosity (or flow time) after addition of 50 µl 5% glucose in water with or without 0 5% Ubiquin All figures are means of 4 separate experiments in different viscosimeters The first column gives the volume of the hyaluronate solution in the viscosimeters before adding the 50 µl-sample

	Glucose	Ubiquin + glucose	Calculated decrease due to dilution
250 µl	83 8	83 6	83 3
500 µl	89 2	89 9	90 9

In several experiments with 4 viscosimeters having various flow times, the reduction in viscosity appeared the same from addition of ubiquin along with glucose as when only glucose solution was added in the same volume The decrease in viscosity was found experimentally to be close to the figures calculated from the concept of simple dilution as the cause of the decrease in viscosity (table 3) The concentrations of ubiquin in the viscosimeters were 0 505 and 0 275 mM, respectively, in the experiments with 250 µl and 500 µl samples

In experiments on 3 rabbits, 5 guinea pigs and 5 rats we found no alterations produced by ubiquin given intravenously in doses of 10 mg/kg on blood pressure or on frequency and volume of respiration Equivalent amounts of histamine and 5 hydroxytryptamine caused significant alterations

Discussion

The substance ubiquin ® should have the characteristics of a polyelectrolyte, since it is described as a polymer of the chloride of oligo N methylmorpholinium propylene-oxide The unit structure is shown in fig 2 The mean molecular weight is said to be 1650, giving a mean number of 10 units in each molecule (PABST 1961) It has no chemical similarities to the enediols of DAUBENMERKL (1953) mentioned earlier

Non-diffusible polyelectrolytes can associate with oppositely charged ions and thus retard absorption HIGUCHI *et al* (1954) attempted to establish whether or not the absorption of similarly charged ions was enhanced by polyelectrolytes They considered the problem theoretically on the basis of the Donnan equilibrium Further, they performed *in vitro* experiments on the diffusion through a semipermeable membrane These *in vitro* studies confirmed the theory that negatively charged diffusible ions, such as salicylate and penicillin, passed through the membrane at a greater rate when negatively charged non-diffusible electrolytes, such as

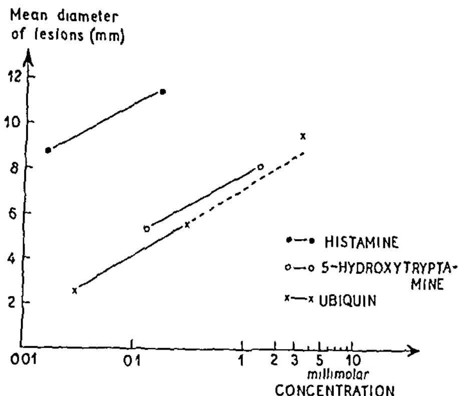


Fig 1 The relationship between molar concentrations of *ubiquin histamine* and 5 hydroxytryptamine and their effects on capillary permeability in *guinea pigs* (se Methods') Abscissa concentration as millimoles (logarithmic scale) Ordinate Mean of the diameters from at least 48 experiments for each value
● —— ● histamine
○ —— ○ 5 hydroxytryptamine
× —— × *Ubiquin* ®

line for ubiquin has therefore been drawn only through the points obtained from the experiments at the two lowest concentrations of ubiquin, the rest of the line being dotted For comparison the log dose response lines for histamine and 5-hydroxytryptamine are shown on the graph Clearly ubiquin is a capillary permeability factor In equimolar concentration it is quantitatively similar to 5-hydroxytryptamine, whereas histamine is 100 times as potent

As described in "Methods", ubiquin was tried on the *guinea pig ileum* for histamine-like action and histamine antagonism Either of these effects was shown in several experiments The sensitivity of the ileum preparation commonly permitted a contraction that was enlarged to 40 mm on the black drum when reacting to a histamine concentration of 1×10^{-4} mM in the Tyrode solution Ubiquin at a concentration of 0 121 mM did not evoke any recordable contraction and did not decrease the histamine contractions to any measurable extent Histamine caused significant contractions at a concentration of 10^{-5} mM

pillary resistance and of other vessels to trauma In the absorption ex periments which involved sampling blood from the ear marginal vein of rabbits we closed the wound in the intervals between sampling by applying cotton wool to the lesion kept in position by a clothes peg Several of the animals developed necroses in this same area during the days after the absorption experiment with ubiquin In numerous absorption experiments performed previously by us such changes have never been recorded

Thus we have been unable to show any absorption-enhancing effect of the drug when given by the intravenous route simultaneously with the subcutaneous administration of sulphacetamide sodium Given locally ubiquin increases the permeability of capillaries and a decrease in vascular resistency may result from intravenous administration This fact may explain the claim that the substance should possess hyaluronidase activity

Summary

Ubiquin ® is the chloride of a polymer of oligo N methyl morpholo nium propylene oxide We were not able to demonstrate any alteration exerted by intravenous ubiquin on rate of absorption of subcutaneously injected sulphacetamide sodium

The substance did not alter the viscosity of potassium hyaluronate or the spreading of fluid in connective tissue Neither histamine like action nor histamine antagonism was found

However the permeability of capillaries to substances of high mole cular weight was increased and the resistance of capillaries to trauma seemed to be lowered

It is concluded that the substance is without any absorption enhancing effect similar in mechanism to that of hyaluronidase

Acknowledgements

Enthusiastic technical assistance from Miss Karin Dyhrfjeld is grate fully acknowledged The substance was put at our disposal by courtesy of Professor Erik Jacobsen

REFERENCES

Dalgaard Mikkelsen S & S A Kvorning Viscosimetric estima on of hyaluron dase *Acta pha* –

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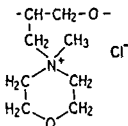


Fig 2 Unit structure of the molecule of ubiquin
With a mean molecular weight of 1650, the number of units in each molecule is ten (PABST 1961)

carboxymethylcellulose, were found in the solution to exert an exclusive effect Later authors failed to show such an effect of a positively charged polyelectrolyte as PVP (polyvinylpyrrolidine) on the absorption of atropine sulphate *in vivo* (SCHRIFTMAN & KONDRITZER 1957)

However, as far as the information is available from the paper of PABST (1961), the tissue clearance test show the rate of absorption of a negatively charged ion ^{131}I, and the polyelectrolyte is positively charged Further, the molecular size of ubiquin (mol wt 1650) does not indicate any prohibition of diffusion through capillary walls Consequently any such mechanism as the above-mentioned cannot explain the results reported. Further, if the mean molecular weight is only 1650, most of the molect eading factor quin at the concentrations used in our experiments Neither did the substance inhibit the effect of histamine on the isolated guinea pig ileum or on the blood pressure of various species of animals It was without effect on the viscosity of a potassium hyaluronate solution The only positive pharmacological effect of ubiquin demonstrated in our investigation was an increase in the permeability of the capillaries This was evaluated by the passage of intravascularly administered Evans blue through the capillaries into the tissue in the areas where ubiquin was injected locally The molar effect on the permeability of capillaries was of the same order as that exerted by 5-hydroxytryptamine

A possible explanation for the results of PABST (1961) would be an enhanced filtration of fluid through the vascular walls in the injection zone when ubiquin is added to the injected solutions containing ^{131}I If oedema fluid is extravasated into the interstitial tissue containing ^{131}I, it may cause a more rapid decrease in radiation as it is measured over the skin, simulating the alterations found when an absorption enhancing agent operates There are no data showing how much radioactivity could be measured in the blood

Our observations indicate that ubiquin may cause a decrease in ca-

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From the Department of Pharmacology, University of Gothenburg, Goteborg Sweden

The Comparative Absorption of Creatinine from »Gitter« Tablets and Control Tablets.

By

Lennart Lundholm & Nils Svedmyr

(Received February 2, 1963)

One goal of current pharmacologic research is a more even and prolonged absorption and blood concentration of drugs achieved by incorporating the active substances in oral preparations giving sustained release Numerous preparations with a reportedly prolonged pharmacological action have been produced DRAGSTEDT (1958) has critically discussed the advantages and drawbacks of the method There are, essentially, two considerations first, the purely technical problem of developing preparations capable of giving, under varying conditions, a uniform and sustained release, and, secondly, the physiological question of the extent to which the released drug will be absorbed in the more distal sectors of the gastro intestinal tract

SJOGREN & FRYKLOF (1960) described a form of "gitter" tablet (Duretter ®, Hässle), in this the active substance is incorporated in a network of plastic from which, through variations in the method of preparation *in vitro*, it can be released at varying rates Below we report a comparative study of the absorption of creatinine as administered in Duretter ® and in conventional tablets Creatinine was used as test substance because of its comparatively rapid absorption, being mostly absorbed within two hours Creatinine absorption, moreover, is relatively complete, amounting to approximately 75% of the amount administered orally Nor does it appear to be metabolized in the organism, for 96 per cent of intravenously administered creatinine has been recovered in the urine (DOMINGUEZ & POMERENE 1945) Finally, since creatinine can be determined with satisfactory accuracy in blood and urine, it is well suited for absorption studies The "gitter" tablets used in this investigation were so constituted that all the creatinine was released within six hours roughly the time required for passage through the small intestine

Jensen, C E The preparation of hyaluronic acid and the determination of the viscosity and optical rotation *Acta Chem Scand* 1953, 7, 603–610
Pabst, H W Über synthetische Diffusionsfaktoren I Mitteilung Chemie und Spreading Förderung *Klin Wschr* 1961, 39, 765–766
Schmidt, A Spreading test in subfascial connective tissue without indicator *Acta pharmacol et toxicol* 1956, 12, 164–170
Schou, J Absorption of drugs from subcutaneous connective tissue *Pharmacol Rev* 1961, 13, 441–464
Schou, J The influence of cortisone on the subcutaneous absorption of drugs *Acta pharmacol et toxicol* 1958, 14 251–264
Schriftman, H & A A Kondritzer Absorption of atropine from muscle *Am J Physiol* 1957, 191, 591–594

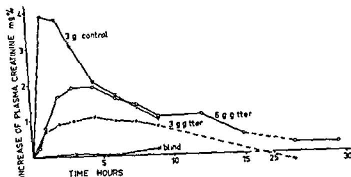


Fig 1 Increase of plasma creatinine level over the basal value in the different experiments

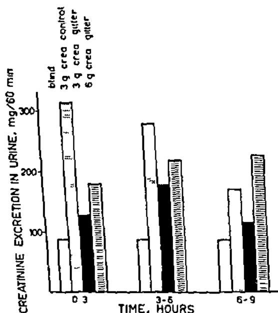


Fig 2. Creatinine excretion in the urine during three hour periods in the different experiments

however, the creatinine contents of the blood and the body fluid were still elevated by about 1 mg% over the basal value Creatinine is evenly distributed through the water phase of the organism (DOMINGUEZ & POMERENE 1945), which constitutes approximately 70% of the body weight in these experiments about 50 kilograms The amount of creatinine absorbed and remaining in the organism after nine hours can therefore

Methods

The experiments were performed on five healthy male students

The subjects came to the Institution in the mornings after fasting since the previous evening Basal blood (20 ml) and urine tests were first made, the subjects being instructed to empty their bladders completely The tablets were then swallowed after which blood samples were taken at 30 minutes 1 hour, 2, 3, 4½ 6 7½ and 9 hours At three hour intervals (0–3, 3–6 and 6–9 hours) the subjects emptied their bladders as completely as possible The urinary volume was measured and from it 50 ml were taken for analysis In the experiments with 6 g creatinine (48 tablets) blood samples were also taken at 12, 15, 26½ and 29½ hours and the urine was collected during the intervals

Each subject drank approximately 300 ml liquid (water or fruitjuice) when swallowing the tablets and the same volume every three hours thereafter A standard meal consisting of two sandwiches and fruitjuice, was taken three hours after administering the tablets and then twice more at three hour intervals The subjects sat quietly throughout the experiments

Each subject underwent three experiments in the first of which he received 24 blank 'gitter tablets, in the second ordinary creatinine tablets and in the third creatinine 'gitter' tablets Three of the five subjects underwent additional experiments in which the dose of creatinine 'gitter tablets was doubled (6 g) The first three sets of experiments were conducted at intervals of about three weeks the last set was about one year later

The blood samples for determining creatinine were centrifuged immediately after being taken, and creatinine was assayed by the method of OWEN *et al* (1954) in the plasma

Results.

Blank tests The purpose of these tests was to ascertain whether the creatinine content of the blood varied during the experiments and to determine endogenous creatinine excretion The creatinine content was in general extremely constant (fig 1) Elimination of endogenous creatinine in the urine was also constant (fig 2), the total creatinine excretion over a nine-hour period averaged 783 mg

Creatinine control experiments Each subject received 24 ordinary tablets containing a total of 3 0 g creatinine Within one hour, as shown in fig 1, the plasma creatinine had reached a peak, on the average 4 mg% above the basal value The creatinine content subsequently fell, at first sharply, but nine hours after beginning the experiment it was still approximately 1 0 mg% above the basal value

During the first three hours the creatinine excretion in the urine was about three times that recorded in the blank tests, but it then showed a gradual fall (fig 2) The total creatinine excretion over a nine hour period was on the average 2 26 g, an increase of 1 48 g over the blank value and equivalent to 49% of the amount administered At the end of nine hours,

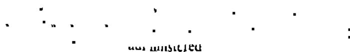
than in the creatinine control experiments A steady maximum of 2 6 mg%, equivalent to an increase of 1 4 mg% over the basal value, was reached 4½ hours after administering the tablets, with then a gradual decline, at the end of nine hours the level was almost identical with that in the control experiments

The creatinine excreted in the urine showed only a slight rise during the first three hours, but in the second three hour period was approximately double that noted in the blank tests (fig 2) The total amount excreted through the kidneys over a nine hour period was 1 24 g, or 0 45 g above that in the blank tests Allowing for the amount absorbed and remaining in the organism, the total creatinine absorption may be estimated at 0 45 plus 0 54 g, or 0 99 g, equivalent to 33%

Creatinine "gitter" tablets in double dose In the experiments already described the absorption of creatinine from "gitter" tablets was less complete than that from control tablets In the next set of experiments it was sought to obtain equal absorption through administering 6 g creatinine in "gitter" tablets, the preceding values having indicated that this dose would provide a total absorption of the same order as that from the control tablets The experimental period was extended to 30 hours in order to make posible determination of total creatinine absorption with greater accuracy

It will be seen from fig 1 that during the first hour the plasma creatinine did not increase more rapidly in the experiments with 48 "gitter" tablets than in those with 24 tablets At the end of two hours however, it had doubled as shown in fig 3, for which the percentage increase in plasma creatinine has been calculated on the basis of the values for 24 "gitter" tablets In these experiments also the maximum concentration was reached after 4½ hours, the creatinine level then gradually declining At 26½ hours and 29½ hours the plasma creatinine was constant at 0 27 mg% over the basal value

Creatinine excretion in the urine showed a constant maximum three to nine hours after



administered

From the difference between the plasma creatinine levels at 9 and 29½ hours (0 75 mg%) the eliminated amount may be estimated at 0 38 g, which is close to the directly determined excess of 0 4 g eliminated in the urine This indicates the reliability of the former mode of calculation The amount of creatinine absorbed at the end of nine hours was estimated at 1 63 g, equivalent to 27% of the amount administered

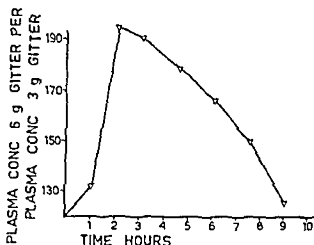


Fig 3 Percentage increase of plasma creatinine concentration at various intervals after administering 6 g compared with 3 g creatinine in "gitter" tablets

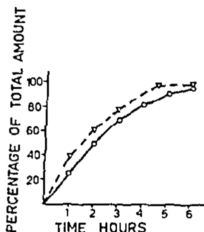


Fig 4 Lower curve creatinine release as percentage of total amount in *in vitro* experiments with "gitter" tablets Upper curve creatinine absorption at various times as percentage of total creatinine absorption

be estimated at 0 5 g (10 mg × 50) It may thus be calculated that a total of 1 48 plus 0 5 g, or 1 98 g creatinine was absorbed, representing 65 per cent of the amount administered For comments on the reliability of these calculations, see below

Creatinine "gitter" tablets In these experiments each subject ingested 24 "gitter" tablets containing a total of 3 g creatinine – The rate of release of creatinine *in vitro* from Duretter (8) is given in fig 4, which shows that after six hours practically all the creatinine had been released The plasma creatinine level rose at a considerably slower rate in these

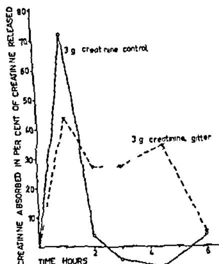


Fig 5 Creatinine absorbed as percentage of creatinine administered (creatinine control tablets) or creatinine released from gitter tablets during one hour (creatinine gitter tablets)

indicates, however, that such an assumption would be erroneous, since in the experiments with 'gitter" tablets the plasma creatinine level continued to rise for 4–5 hours Calculation of the average percentage absorption of creatinine released during each hour from the creatinine 'gitter' tablets yielded the values given in fig 5

The absorption of released creatinine was thus appreciably greater during the first hour than afterwards Considerable amounts of creatinine were nevertheless absorbed throughout the period of 4–6 hours during which the gitter' tablets could be assumed to be passing through the small intestine Judging by the results the absorption of creatinine was more thorough in the proximal than in the distal sectors of the gastro intestinal tract Hence, the explanation of the low creatinine absorption from "gitter" tablets could well be that most of the absorption took place in more distal sectors than did that from the control tablets It was possible, however, to offset in some degree the poorer absorption from "gitter" tablets by increasing the dose of creatinine

The observations suggest that creatinine incorporated in "gitter" tablets was released in the intestine at a rate similar to that observed *in vitro* The technical problem of developing an oral preparation with sustained release thus appears to have been satisfactorily resolved On the other hand, an answer to the physiological question of the extent to which the released substance will be absorbed in distal portions of the

Discussion

In fig 4 the creatinine absorption at given intervals is recorded as a percentage of the total absorption The resulting curve corresponds closely to that depicting the rate of creatinine release from "gitter tablets *in vitro*, showing the intestinal release of creatinine from "gitter' tablets conformed to the time pattern of *in vitro* release

It is evident from fig 1 that in these experiments the creatinine "gitter' tablets did not have a more prolonged action than the creatinine control tablets At the end of nine hours the plasma creatinine concentration was almost identical in the three series of experiments The two types of tablets differed essentially is one respect the concentration curve for the control tablets exhibited a peak, whereas that for the creatinine "gitter tablets had no peak With the "gitter" tablets, however, the maximum plasma creatinine concentration was only 25% of that in the creatinine control experiments It was nevertheless possible, by doubling the dose of creatinine "gitter" tablets, to double the maximum concentration in the blood The therapeutically significant conclusion to be drawn from fig 1 is that it should be possible with "gitter" tablets to obtain a more even blood concentration over a given period than with control tablets If a drug whose absorption behavior is like that of creatinine, has a thera peutic plasma concentration close to the toxic, the use of "gitter" tablets could prove advantageous a peak concentration associated with side effects might thereby be avoided

On the other hand, it will be seen from fig 1 that with creatinine "gitter" tablets the maximum concentration in the blood was not reached until after three to four hours To ensure more rapid action it might therefore be advisable to "prime" the organism by administering part of the substance in rapidly absorbable form

The fact that "gitter" tablets did not have a prolonged action in these experiments is doubtless due to the poorer absorption of creatinine amounting to only about 50% of that from the control tablets DOMINQUES & POMERENCE (1945) in their experiments noted that creatinine absorption had practically ceased after two hours, by which time about 75% of the dose administered had been absorbed The absorption of creatinine in our control experiments, calculated from the change in plasma concentration and from the amount excreted with the urine and expressed as a percentage of the amount administered, is evident from fig 5 The absorption had almost ceased after one hour, and the remaining 25–30% in the intestine was not absorbed It would seem reasonable to assume that creatinine absorption was limited to the upper portions of the intestine and failed to occur in the distal sectors A study of fig 1

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From the Departments of Pharmacology and Pathology
B J Medical College, Poona 1, (India)

Protection by Indigenous Drugs Against Hepatotoxic Effects of Carbon Tetrachloride in Mice

By

G V. Joglekar, G K Chitale & J H Balwani

(Received February 18 1963)

In recent years much work has appeared in the literature about the induction of liver damage by carbon tetrachloride and protection against it by a wide variety of agents, such as antihistaminics, phenothiazines, quinine and procaine (REES 1962, FIUME *et al* 1961) LIV 52* a proprietary medicine consisting of indigenous drugs, has been claimed to be effective in hepatic disorders (SHETH *et al* 1960, PATRAO 1957, SULE *et al* 1956)

In our work we proposed to study the protection offered by promethazine (phenergan ®), chlorpromazine (largactil ®) and LIV 52 against the effects of carbon tetrachloride in mice

Materials and Method

We used 100 albino mice, of average weight range 25–40 g For the short term study 50 male mice were divided into five equal groups One group was kept as a control receiving no treatment Animals in each of the other four groups were given carbon tetrachloride 0 1 ml orally by intragastric tube One group out of these four was given no protective agent

The remaining three groups received phenergan elixir (1 mg/mouse) largactil (0 4 mg mouse) or LIV 52 Pediatric drops (0 4 ml i e 25 mg mouse) immediately after (i e 0 hrs) and again 4 hrs after the administration of the carbon tetrachloride

* Product of the Himalaya Drug Co Bombay

gastro intestinal tract still present major difficulties Although it may be possible, for a given substance, to combine a rapidly absorbed and a slowly absorbed form in a single preparation that will on the average provide, a constant and even blood concentration, there still remain important questions about the extent of individual deviations from the mean and the degree to which drugs vary in their absorbability If the principle of sustained release is to be successfully applied to drugs, it seems essential to undertake careful studies of the absorption and blood concentration of each individual drug, since the factors governing absorption in the distal segments of the small intestine are imperfectly understood

Summary.

Creatinine tablets of two types were administered by mouth to each of five subjects, after which the plasma creatinine concentration and the excretion in the urine were studied The test preparation consisted of "gitter" tablets (Duretter ®, Hassle) in which the creatinine was incorporated in a network of plastic for sustained release Conventional creatinine tablets were used as controls The *in vivo* rate of absorption of creatinine from the "gitter" tablets was similar to the *in vitro* rate of release from corresponding tablets The total creatinine absorption from 'gitter tablets, however, was only half that from the control tablets presumably because from the former the process of absorption occurred largely in the more distal sectors of the small intestine The plasma creatinine concentration rose more slowly and showed a more regular course from "gitter" tablets than from the control tablets

Acknowledgements

Financial support has been given by Hassle drug Co Goteborg

REFERENCES

Dominguez R & E Pomerene Calculation of the Rate of Absorption of ... *Proc Soc Exp Biol & Med* 1945 60 173 181

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Table 3

Histological changes in Liver after 72 hrs
50 ♂ mice, five groups of 10 Numbers showing change

	Control	CCl_4	CCl_4 & Phenergan	CCl_4 & Largactil	CCl_4 & Liv 52
Diffuse necrosis	Nil	10	10	10	10
Hydropic degeneration	Nil	3	5	4	2
Fatty degeneration	2	7	7	6	9
Hemorrhages	Nil	5	2	4	6
Congestion	3	10	8	7	9

Table 4

Histological Changes in Liver after 21 days
50 ♀ mice five groups of 10 Numbers Showing change

	Control	CCl_4	CCl_4 & Phenergan	CCl_4 & Largactil	CCl_4 & Liv 52
Areas of Active Necrosis	Nil	10	8	10	2
Chronic cell infiltration	Nil	10	9	8	Nil
Localised Regenerative activity	Nil	8	6	8	2
Generalised Regenerative activity	Nil	Nil	Nil	Nil	8
Lobulation	Nil	8	6	7	1
Fatty degeneration	3	7	4	4	5
Reticulum	–	Normal	Normal	Normal	Normal

The remaining three groups in which the protective agents were given after the carbon tetrachloride showed almost similar picture

21 days' study In the carbon tetrachloride group (fig 1) the areas of necrosis were being gradually replaced by bands of cells consisting of histiocytes and other chronic inflammatory cells, including plasma cells Certain areas still showed evidence of active necrosis, whereas the intervening cells showed regenerative activity The [illegible] …atory cells in the necrosed area and regenerative activity in the surrounding cells However, the changes were little different from those noticed with carbon tetrachloride alone

On the other hand in the LIV 52 group (fig 4) regenerative activity was marked even in the previously necrosed areas, and replacement by

Table 1

Group (50 ♂ mice)	No of animals living after 72 hrs	% mortality
Blank Control	10	0
Carbon tetrachloride Control	2	80
Carbon tetrachloride & Phenergan	2	80
Carbon tetrachloride & Largactil	2	80
Carbon tetrachloride & LIV 52	7	30

Table 2

Group (50 ♀ mice)	No of animals living after 21 days	% mortality
Blank Control	10	0
[illegible]	1	90
[illegible]	0	100
[illegible]	2	80
[illegible]	7	30

The animals were observed for 72 hrs Those surviving at the end of the observation period were killed and subjected to histological examination

A similar study has been intended on 50 female mice However, at the end of 72 hrs they were all alive, and it was therefore decided to extend the study by repeated administration of carbon tetrachloride

Distributions into groups and the administration of carbon tetrachloride and other drugs was done in the same way as in the short term experiment In this study carbon tetrachloride was administered five times at the interval of three days for 21 days To the animals in the three groups, 0 hrs and 4 hrs after each carbon tetrachloride administration, phenergan, largactil or LIV 52 was given orally in the doses already mentioned The livers of those dying during the study period and those surviving to the end were examined histologically

Results.

In table 1 and 2 are given the figures for survival in the two experiments Table 3 and 4 summarize the results of the histological study

72 hrs study In the carbon tetrachloride group diffuse necrosis of the liver parenchyma was noticed as acidophilic pink areas However, it was especially marked in the layers of cells surrounding the central vein

Other areas showed degenerative changes as hydropic and fatty degeneration Haemorrhages were also present in few areas

chronic inflammatory cells was almost negligible The liver pictures showed striking resemblances to those of the control group

Reticulum staining in all the groups did not reveal any abnormality

On the basis of the experience gained during this study, a long term investigations on rats is in progress

Comments and Methods

The present day drug treatment of liver cirrhosis is still in the experimental stage No drug can prevent the cirrhotic change in the hepatic tissue once it begins

Experimental liver cirrhosis is commonly produced by administering carbon tetrachloride by various routes REES (1962) has reported the protective effects of certain phenothiazine compounds and other agents On the other hand, chlorpromazine, a phenothiazine derivative, has produced liver toxicity in certain clinical cases (KELSEY *et al* 1955, COHEN *et al* 1955, ISAACS *et al* 1955)

An indigenous proprietary medicine, LIV 52, containing reputed hepatic stimulants (KIRTIKAR & BASU 1933) was clinically tested and showed some value The LIV 52 drops contain *capparis spinosa* 1 67%, *tamarix gallica* 0 42%, *cassia occidentalis* 0 42%, *terminalia arjuna* 0 84%, *achilea millefolium* 0 42%, *cinchorium intybus* 1 67% and *solanum nigrum* 0 84%

In the investigation we have studied the effects of promethazine (phenergan), chlorpromazine (largactil) and LIV 52 on carbon tetrachloride induced liver damage in mice The short study of male mice for 72 hrs (table 1) clearly shows that the percentage mortality with LIV 52 is markedly less than that in the other groups Phenergan and largactil failed to protect the mice against carbon tetrachloride, since the same number of deaths occurred in all the three groups Histologically, however, no clear protective effects of LIV 52 were noted

When a similar study was carried out on female mice, no death occurred in 72 hrs GYORGY *et al* (1946) have observed a similar longer survival and less hepatic damage in female rats than in male ones after carbon tetrachloride treatment When this study was [illegible] after the repeated adminis[illegible] mortality with LIV 52 wa[illegible] hrs (table 2) and considerably less than in the other groups Phenergan and largactil again failed to protect the animals

Histological study of these groups, showing early cirrhotic change as evidenced by infiltration of chronic inflammatory cells, suggestion of lobulation and localised regenerative activity, clearly indicates that

Fig 1. Carbon-tetrachloride effect "Pro-

... Phenergan

chronic inflammatory cells was almost negligible The liver pictures showed striking resemblances to those of the control group

Reticulum staining in all the groups did not reveal any abnormality

On the basis of the experience gained during this study, a long term investigations on rats is in progress

Comments and Methods

The present day drug treatment of liver cirrhosis is still in the experimental stage No drug can prevent the cirrhotic change in the hepatic tissue once it begins

Experimental liver cirrhosis is commonly produced by administering carbon tetrachloride by various routes REES (1962) has reported the protective effects of certain phenothiazine compounds and other agents On the other hand, chlorpromazine, a phenothiazine derivative, has produced liver toxicity in certain clinical cases (KELSEY *et al* 1955, COHEN *et al* 1955, ISAACS *et al* 1955)

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promethazine and chlorpromazine were both unable to stop the changes occurring after carbon tetrachloride administration In the LIV 52 group the liver picture had a remarkable similarity to that of control livers there being no areas of chronic cell infiltration and the necrosis that might have resulted was completely replaced by active regeneration spread throughout This suggests that LIV 52 can possibly prevent liver damage and may even be useful in preventing further fibrotic changes

Since our study was only for of limited duration, reticulum staining showed no change, which requires more than 8 wks to become obvious (FIUME *et al* 1961, WAHI *et al* 1956)

The mechanism of this protective effect of LIV 52 remains unexplained, but the results have encouraged us to pursue a long term study

Summary

Protective effects of LIV 52 – an Indian indigenous proprietary medicine – were noticed against carbon tetrachloride in mice both in a 72 hrs and in a 21 days study Histological changes due to carbon tetrachloride were also prevented

Promethazine and chlorpromazine showed no protection against carbon tetrachloride as evidenced by similar percentage mortality and the nature of hepatic damage

Acknowledgements

We thank Dr F J Mendonca, Dean, B J Medical College, for his encouragement Phenergan and largactil were supplied by May & Baker (Ltd) Bombay, India

REFERENCES

Cohen I M & J D Archer Liver function and hepatic complications in patients receiving chloropromazine *J A M A* 1955 159 99 101

Fiume L & G Favilli *Inhibition of Experimental Cirrhosis by Carbon* ... treatment with Aminoacetonitrile *Nature*

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Sheth S C, B J Northover, N S Tibrewala U R Warerkar & V S Karande Therapy of cirrhosis of liver & liver damage with indigenous drugs – experimental & clinical studies *Indian J Pediat* 1960 27, 204-211

Sule C R, P M Sathe M C Koshy & M S Deshpande Ascitis due to Liver deficiency treated with an indigenous drug *The Indian Practitioner* 1956 9 357 362

Wahi P N H D Tandon & T P Bharadwaj Adrenal Cortex and hepatic cirrhosis *A M A Arch Path* 1956 62 200 214

Acta pharmacol et toxicol 1963, 20, 80–89

From the Department of Pharmacology, University of Copenhagen
(Professor Knud O Møller, M D)

The Lethal Action of Morphine and Nalorphine given jointly to Morphine Tolerant and Non-Tolerant Rats

By

Torkell Johannesson and Kirsten Milthers

(Received March 20 1963)

Although nalorphine is an effective antidote for otherwise fatal doses of morphine when central depression is the predominant toxic manifestation, it is relatively ineffective when convulsions are the significant feature (WOODS 1956) In mice and rats nalorphine does not protect against the convulsant effect of morphine (KOPPANYI & KARCZMAR 1953)

In lethal or other extremely large doses nalorphine may cause excitation and convulsions in many species, *e g* in monkeys (IRWIN & SEEVERS 1954) and in rabbits (LAVIKAINEN & MATTILA 1959), resembling those seen after similar doses of morphine Recently JÓHANNESSON (1962) has suggested that excitation as seen in rats after large doses of morphine may be caused by depressed cholinesterase activity in the brain Nalorphine inhibits cholinesterases to the same or an even greater extent than morphine (BLOHM & WILLMORE 1951, FOLDES *et al* 1959) We have therefore advanced the idea that the excitatory effects of morphine and nalorphine are of the same origin Further, if nalorphine fails to antagonize the cholinesterase inhibition caused by morphine, the lack of antagonism of nalorphine to morphine excitation and lethality in the rat would be explained

Our intention in the experiments recorded here was, first to investigate whether the convulsant and lethal effects of morphine to morphine-tolerant and non-tolerant rats could be increased by simultaneous administration of nalorphine We determined not only the lethal doses, but also the concentrations of morphine and nalorphine in the brain It was therefore possible to estimate whether the concentrations of these compounds in the t

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brain, because AXELROD (1956) has demonstrated *in vitro* a decreased N demethylation of morphine, pethidine and hydromorphone by liver microsomes from morphinetolerant rats and related the development of tolerance to the decreased capacity for N demethylation

Experiments and Methods

Adult male albino rats (125–240 g) were used They were maintained under constant environmental conditions with free access to water and a commercial food preparation Morphine tolerance in the rats was induced as previously described by JÓHANNESSON (1962) Tolerant rats were put on experiments 17–20 hours after the last daily dose of morphine Normal rats are referred to as non tolerant

Morphine chloride (Ph Dan 1948) and nalorphine chloride (Anarcon ®) in the form of 100 mM aqueous solutions were injected intraperitoneally Morphine was

[illegible]

... ic injections Their brains (including the brain stem and the cerebellum) were removed and analysed for morphine and nalorphine Further, the amounts of normorphine formed from these substances *in vivo* were determined

When both drugs were given to the same animal the morphine was injected first and then the nalorphine immediately from another syringe The brains of the rats that had received morphine + nalorphine regardless of whether they died spontaneously or were killed were removed and their contents of morphine, nalorphine and normorphine were determined

Six non tolerant and two tolerant rats were killed without any previous administration of drugs Their brains were removed and homogenized with an appropriate volume of 0 9% sodium chloride solution in a POTTER & ELVEHJEM homogenizer By means of an ice bath the temperature was kept low before, during, and after homogenization Cholinesterase activities were then determined in the total brains of both tolerant and non tolerant rats The activities ranged between 0 95 and 1 1 mol ...

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... e activities were then determined and expressed as percentages of the control values (for details see JÓHANNESSON & LAUSEN 1961)

Determinations of morphine nalorphine, and normorphine were performed as described by MILTHERS (1961) The average recovery was about 97%, 97% and 72%, respectively In calculating the concentrations of normorphine corrections were made for the low percentage recovery The lowest measurable amounts are about 1–2 μg/g brain

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Table I

The concentrations of morphine nalorphine and normorphine in the brain of morphine [illegible]

third column

Drugs injected	Dose mg/kg	Minutes after inj	[illegible] phine	[illegible] phine	[illegible] phine	[illegible] phine	[illegible] phine	[illegible] mor phine
Morphine	500	15	13 0		2 0	17 0		n m
		15	12 0			11 9		
		15	15 5		1 0	9 1		n m
		15	12 2			16 0		
		30	17 2		1 8			
		30	11 7					
		30	15 6		1 2			
		30	20 0					
	600	15	20 5		1 4	19 0		1 0
		15	27 6			16 6		
		15 d	20 5		1 2			
		15 d	23 3					
		15	31 9		1 2			
		15	15 3					
		30	32 6		1 5	22 7		n m
		30	50 3			30 9		
		30				10 8		n m
		30				24 4		
		30				19 0		1 0
		30				24 1		
Nalorphine	200	15		38 6	1 2		4 4	4 5
		15		34 2			41 9	
		15		19 4	1 3		22 6	1 0
		15		16 9			26 1	
		15		15 2	1 0		18 4	1 0
		15		19 9			29 0	n p
		30		6 9	n m		22 1	n m
		30		11 0			17 3	
		30		6 6	n m		17 1	1 9
		30		13 5			4 3	
		30		24 4	1 0		18 0	n p
		30		4 0				
		30		14 7	n p			
		30		19 7	n.p			
Morphine plus Nalorphine	500 plus 200	29 d	17 3	17 1	3 0			
		30 d	17 8	26 7				
		25 d	16 6	26 6	4 4			
		24 d	15 6	43 9				
		37 d	18 8	30 7	1 1			
		30 d	14 8	24 4				
		25				12.6	6 3	1 4
		25				16 9	19 4	

SEN HOLM (1961) In our experiments the total reaction volume was 50 ml and measurements were made at pH 7 4 and 38 4° The cholinesterase activity is expressed as micromoles liberated acid per minute per gram tissue

For titration we employed 10 mM solution of NaOH Acetylcholine iodide was applied as a substrate at molarity x10 5

Results.

Injections of 50 or 100 mg/kg nalorphine + 500 mg/kg morphine were generally not lethal to the non tolerant rats Injections of 500 mg/kg morphine + 200 mg/kg nalorphine were lethal to the non tolerant rats, which died in convulsions 24–37 minutes after the injections Thirteen tolerant rats received 600 mg/kg morphine + 200 mg/kg nalorphine Eight of them died in convulsions 15–40 minutes (average of 23 minutes) after the injections, whereas the remaining five survived for at least 24 hours and were eliminated from the experiment Eight tolerant rats received 500 mg/kg morphine + 200 mg/kg nalorphine One of them died in convulsions 20 minutes later, but the remaining seven rats survived for at least several days and were eliminated from the experiment For comparison four more tolerant rats received these doses of morphine and nalorphine and were killed 25 minutes after the injections, which was approximately at the mean time of death of the tolerant rats given 600 mg/kg morphine + 200 mg/kg nalorphine

I *Morphine, Nalorphine and Normorphine in the Brain*

The results are shown in table 1

A Injections of morphine alone In the brains of rats receiving 500 mg/kg morphine, the average concentration of the drug 15 minutes after the injections were approximately the same in the tolerant and in the non tolerant rats, about 13–14 µg/g brain Thirty minutes after the injections of 500 mg/kg of morphine the average concentration in the non tolerant rats was 16 µg/g brain When the rats had received 600 mg/kg morphine, we found on an average of 23 µg/g brain in the non tolerant rats 15 minutes after the injections In the tolerant rats receiving 600 mg/kg morphine, the average concentrations were 18 µg/g and 22 µg/g brain 15 and 30 minutes after the injections respectively

B Injections of nalorphine alone As previously mentioned nalorphine given separately was never lethal to the rats It should however, be mentioned that the rats showed excitation, and some of them even developed convulsions 10–20 minutes after being injected This

No statistically significant differences were found between the nalorphine concentrations in the brains of the tolerant and the non tolerant rats given 500 mg/kg morphine + 200 mg/kg nalorphine ($P > 0.05$) On the other hand, the nalorphine concentrations in the brain of the tolerant and non-tolerant rats, given the doses mentioned of morphine and nalorphine (500 mg/kg + 200 mg/kg), were found significantly different from the nalorphine concentrations in the brain of the tolerant rats given 600 mg/kg morphine + 200 mg/kg nalorphine ($t = 3.7, f = 17, P < 0.01$)

D Normorphine Only small amounts of normorphine were found, so small indeed, that they could not always be determined The highest concentration was found in the brain of the non tolerant rats receiving morphine + nalorphine

2 *Inhibition of Cholinesterase Activity in Brain Homogenates by Morphine, Nalorphine and Normorphine*

The results are given in fig 1

Of the three drugs employed, morphine inhibits brain cholinesterase activity least When the drugs were added to the reaction solution at concentrations of 50 µM, morphine reduced cholinesterase activity in the non tolerant rats to 90% of the control value, whereas nalorphine and normorphine reduced the activity to about 70% and 60%, respectively When morphine, nalorphine and normorphine were employed as 100 µM solutions the remaining cholinesterase activities in the non tolerant rats were about 60% 50% and 40% respectively When morphine and nalorphine were added simultaneously, each at 50 µM concentrations, brain cholinesterase activities were reduced to 55% of the control value

In brains of two tolerant rats, morphine and nalorphine (50 µM) had approximately the same inhibitory effect as in brains of non tolerant rats When added simultaneously, each at 50µM concentrations, the results, as was found in experiments with non tolerant rats, indicated an additive effect of morphine and nalorphine as inhibitors of brain cholinesterase activity and astonishing

Discussion and Conclusions

The results indicate that *more nalorphine* is found in the brain of non-tolerant rats when it is given together with morphine than when it is given alone and that morphine is found in the same amounts in the brain, whether it is given alone or with nalorphine In the tolerant rats, the amounts of nalorphine increased in the brain when the morphine dose was increased We have no explanation for these remarkable findings How-

Drugs injected	Dose mg/kg	Minutes after inj	phine	phine	mor- phine	phine	phine	mor phine
	500 plus 200	25				19 0	23 5	n p
		25				20 2	21 4	2 1
		20 d				24 1	42 0	
Morphine plus Nalorphine	600 plus 200	18 d				18 8	38 6	2 3
		15 d				20 6	35 4	
		24 d				16 8	34 7	1 2
		22 d				13 3	48 2	
		39 d				24 7	36 7	1 0
		24 d				12 9	45 3	
		17 d				18 6	71 2	1 0
		21 d				14 4	53 1	

behaviour was transient, and after about 30 minutes the rats were apparently normal

In the brains of rats receiving 200 mg/kg nalorphine the amounts of the drug 15 minutes after injection were found to be of the same order of magnitude in both tolerant and non tolerant rats, on average 24 µg/g brain After 30 minutes the average concentration of nalorphine was found to be smaller in both groups of rats, on average 13 µg/g in the non tolerant and 16 µg/g in the tolerant rats

C Simultaneous injections of morphine and nalorphine In the *brain of the non-tolerant rats receiving 500 mg/kg morphine* + 200 mg/kg nalorphine, the amounts of morphine at the time of death, 24–37 minutes (average of 29 minutes) after the injections, were found to be *nearly* the same as what could be expected when morphine had been injected alone The average morphine concentration was 17 µg/g brain Nalorphine was found at much higher concentrations, an average of 28 µg/g brain, and this amount greatly exceeded what could be expected when nalorphine was given alone

When 500 mg/kg morphine + 200 mg/kg nalorphine were administered to the tolerant rats, the average concentrations 25 minutes after the injections (1 of the rats died after *20 minutes) were 19 µg/g morphine and 23* µg/g nalorphine When morphine was given to the tolerant rats in amounts of 600 mg/kg simultaneously with 200 mg/kg nalorphine, the average concentrations at the time of death, 15–40 minutes after the injections, were 18 µg/g morphine and 45 µg/g nalorphine, a remarkably high concentration of the drug

environment that may determine cholinesterase activity in the central nervous system *in vivo* (e g substrate concentration and electrolytes)

In our *in vitro* experiments we used 10^{-5}M acetylcholine as a substrate A further increase of inhibition would have been found had we used a lower substrate concentration In this connection it is of considerable interest, that according to JENSEN HOLM (1961) there is no reason to believe that the physiological concentration of acetylcholine in the brain would exceed 10^{-6}M For this reason the inhibition caused by the concentrations of morphine and nalorphine found in the brain *in vivo* could be higher than what we found *in vitro*

As mentioned above, all the non tolerant rats receiving 500 mg/kg morphine plus 200 mg/kg nalorphine died in convulsions within 37 minutes Out of 12 tolerant rats given these amounts of morphine and nalorphine 1 died after 20 minutes, 4 were killed after 25 minutes, and the remaining 7 rats (not included in table 1) all survived for at least several days The concentrations of morphine and nalorphine in the brains of the tolerant and the non tolerant rats given these doses were not significantly different Out of a total of 13 tolerant rats given 600 mg/kg morphine plus 200 mg/kg the 8 rats incorporated in table 1 died within 39 minutes, whereas the remaining 5 rats survived for at least 24 hours The amounts of morphine in the brain of rats in this group did not differ from those in the brain of rats in other groups given 500 mg/kg morphine, and the contents of nalorphine were significantly higher than in any other group (table 1 cf results) The demonstrated increased tolerance to the lethal and convulsant action of morphine and nalorphine must therefore be connected with the fact that the morphine tolerant rats have developed a *tolerance to nalorphine* that makes it possible for them to survive higher concentrations of nalorphine in the brain

JOHANNESSON (1962) found that morphine inhibits brain cholinesterase activity to the same extent in morphine tolerant and non tolerant rats In our [illegible]
activ [illegible]
and [illegible] cholinesterase activity has been found to be the same in homogenates of brain from tolerant and non tolerant rats (JÓHANNESSON 1962) It must therefore be assumed that the observed tolerance to the lethal and convulsant action of morphine and nalorphine described above was not caused by a decreased ability of morphine or nalorphine to inhibit brain cholinesterases or by a decreased ability of the brain cells to hydrolyse acetylcholine

According to MILTHERS (1962) it is likely that normorphine, being a metabolite of morphine and nalorphine, may be formed in the brains of rats Our experiments show, however, that normorphine is produced from

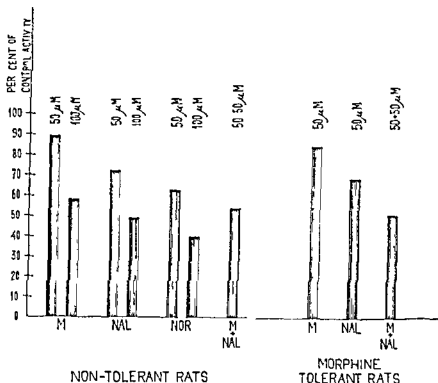


Fig 1 Inhibition of cholinesterase activity in homogenates

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ever, it seems likely that morphine may interfere with the elimination of nalorphine

Intraperitoneal administration of 500 mg/kg morphine or 200 mg/kg of nalorphine to the rats was not lethal in our experiments, but simultaneous administration of the two drugs in these doses was lethal to the non tolerant rats This is probably because the lethal effect of morphine was added to that of nalorphine Further, our results indicate that the drugs are additive in inhibiting cholinesterase activity in homogenates of rat brains

In non-tolerant rats receiving morphine plus nalorphine the mean concentrations of morphine and nalorphine in their brains at death were, expressed in molarity, about 50 μM and 80 μM, respectively Our *in vitro* experiments suggest that brain cholinesterase activity in the rats might have been depressed at least 50% at the time of death (fig 1), and the lethal convulsions in the rats may therefore be correlated with depressed cholinesterase activity in the brain It should, however, be emphasized that we have only an imperfect knowledge about the physiological

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In our *in vitro* experiments we used 10^{-5}M acetylcholine as a substrate A further increase of inhibition would have been found had we used a lower substrate concentration In this connection it is of considerable interest, that, according to JENSEN HOLM (1961), there is no reason to believe that the "physiological" concentration of acetylcholine in the brain would exceed 10^{-6}M For this reason the inhibition caused by the concentrations of morphine and nalorphine found in the brain *in vivo* could be higher than what we found *in vitro*

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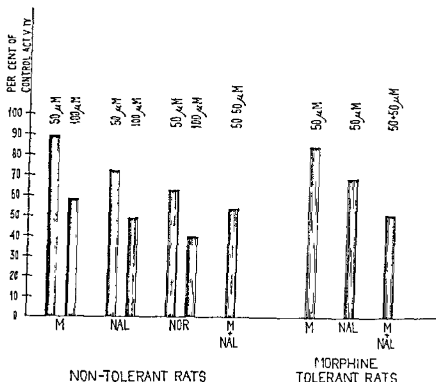
JÓHANNESSON (1962) found that morphine inhibits brain cholinesterase act[illegible]

[illegible] extent in homogenates of brains from tolerant and non tolerant rats, and cholinesterase activity has been found to be the same in homogenates of brain from tolerant and non-tolerant rats (JÓHANNESSON 1962) It must therefore be assumed that the [illegible] tole[illegible]

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REFERENCES

Axelrod, J Possible mechanism of tolerance to narcotic drugs *Science* 1956, 124 263–264

Blohm, T R & W G Willmore Effects of N allylnormorphine on cholinesterases *Proc Soc Exp Biol & Med* 1951, 77, 718–721

Foldes, F F, E G Erdös, N Baart, J Zwartz & E Zsigmond Inhibition of human cholinesterases by narcotic drugs and their antagonists *Arch Int Pharmacodyn* 1959, 120, 286–291

Irwin, S & M H Seevers Acute and antagonistic effects of nalorphine in the monkey *Fed Proc* 1954, 13, 369

384–394

Jóhannesson, T Morphine as an inhibitor of brain cholinesterases in morphine tolerant and non tolerant rats *Acta pharmacol et toxicol* 1962, 19, 23–35

Jóhannesson, T & H H Lausen Clorpromazine as an inhibitor of brain cholinesterases

Kor

Lav

Milthers, K Normorphine, nalorphine and morphine Quantitative separation and determination of small amounts in blood and tissues *Acta pharmacol et toxicol* 1961, 18, 199–206

Milthers, K The *in vivo* transformation of morphine and nalorphine into normorphine in the brain of rats *Acta pharmacol et toxicol* 1962, 19, 235–240

Woods, L A The pharmacology of nalorphine (N allylnormorphine) *Pharmacol Rev* 1956, 8, 175–198

morphine to a greater extent in the non tolerant than in the tolerant rats This is in agreement with the *in vitro* experiments of AXELROD (1956), showing a decreased N-demethylation of morphine by liver microsomes from tolerant rats Whether or not this reduced N demethylation of morphine in the tolerant rats may be causally connected with the develop ment of tolerance, as suggested by AXELROD (1956), is still open to dis cussion. However, in our experiments, nalorphine is not de alkylated to a lesser degree in tolerant rats, further, according to our own (unpublished) results, morphine tolerant rats are also tolerant to the lethal action of normorphine

Summary.

1 Simultaneous intraperitoneal injections of morphine and nalorphine might induce convulsions and death in rats, whereas morphine, or nalor phine, given separately had no lethal action in our experiments Morphine tolerant rats showed increased tolerance to the combined lethal action of morphine and nalorphine

2 At high molarities morphine and nalorphine were potent inhibitors of brain cholinesterase activity in tolerant and non tolerant rats The results indicate that morphine and nalorphine were additive in inhibiting brain cholinesterase activity and that the combined lethal action of morphine and nalorphine could be explained by depressed cholinesterase activity in the brain

3 The demonstrated increased tolerance in the morphine tolerant rats was not caused by a decreased ability of these drugs to inhibit brain cholinesterases or by a decreased ability of the brain cells to hydrolyse acetylcholine It was, however, primarily connected with increased tole rance to nalorphine

4 The results indicate that more nalorphine was found in the brain when it was given with morphine than when given alone It is therefore suggested, that morphine may interfere with the elimination of nalorphine

5 Normorphine, transformed from morphine *in vivo*, was found in lower amounts in the brain of tolerant than of non tolerant rats However, when normorphine was formed *in vivo* from nalorphine, no such difference was found

Acknowledgements

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REFERENCES

Axelrod, J Possible mechanism of tolerance to narcotic drugs *Science* 1956, 124 263–264

Blohm, T R & W G Willmore Effects of N allylnormorphine on cholinesterases *Proc Soc Exp Biol & Med* 1951, 77, 718–721

Foldes, F F, E G Erdös N Baart, J Zwartz & E. Zsigmond Inhibition of human cholinesterases by narcotic drugs and their antagonists *Arch Int Pharmacodyn* 1959, 120, 286–291

Irwin, S & M H Seevers Acute and antagonistic effects of nalorphine in the monkey *Fed Proc* 1954, 13, 369

Jensen Holm, J The cholinesterase activity, alone and the presence of inhibitors, at low substrate concentrations *Acta pharmacol et toxicol* 1961, 18, 379–397

[illegible]
384–394

Jóhannesson, T Morphine as an inhibitor of brain cholinesterases in morphine tolerant and non tolerant rats *Acta pharmacol et toxicol* 1962, 19, 23–35

Jóhannesson, T & H H Lausen Clorpromazine as an inhibitor of brain [illegible]

Kop [illegible]

Lavi [illegible]

Milthers, K Normorphine, nalorphine and morphine Quantitative separation and determination of small amounts in blood and tissues *Acta* [illegible]

Woods, L. A The pharmacology of nalorphine (N allylnormorphine) *Pharmacol Rev* 1956, 8, 175–198

Acta pharmacol et toxicol 1963, 20, 90–96

From the Pharmacology Department, Leo Pharmaceutical Products, Ballerup, Denmark

Monoamine Oxidase Inhibition and Sensitivity of the Nictitating Membrane to Noradrenaline

By

H.-H. Frey

(Received March 19, 1963)

In 1952 BURN & ROBINSON showed that chronic denervation of the nictitating membrane in cats led to a progressive loss of monoamine oxidase (MAO) activity in the organ. There was a good correlation between the degree of enzyme depletion and the response of the nictitating membrane to adrenaline and noradrenaline. Since MAO was at that time commonly believed to be the enzyme responsible for the metabolic degradation of catecholamines, the authors arrived at the conclusion that the enhanced sensitivity of the denervated organ was the consequence of the diminished amount of active enzyme, that is, a slower metabolic inactivation. The well-known inhibitory effect of cocaine on MAO (PHILPOT 1940) fitted into this picture.

In later years it was shown, mainly by AXELROD *et al.* (1960, 1961), that catecholamines are inactivated primarily by another enzyme, O-methyltransferase, and more or less specific tissue binding, MAO playing a minor and indeed doubtful role. It was further shown that cocaine displays its sensitizing effect for catecholamines in doses so small as hardly to have an inhibitory effect on MAO (KRAUSE 1961). Numerous experiments by several authors failed to show any sensitizing effect of the MAO inhibitor iproniazid on the reactions of the nictitating membrane (GRIESEMER *et al.* 1953, BURN *et al.* 1954, BALZER & HOLTZ 1956, KAMIJO *et al.* 1956, SCHMITT & GONNARD 1956), an isolated preparation of the rabbit aorta (GRIESEMER *et al.* 1955) or the vascular musculature of the isolated rabbit ear (KOBINGER & FRIIS 1961) to adrenaline and noradrenaline. This work was done in acute experiments, *i. e.* the reactions to catecholamines were tested before and up to 6 hours after the injection of iproniazid. Since iproniazid has an adrenolytic effect of its own (GRIESEMER *et al.* 1955), and since there were high concentrations in the organism and the isolated organs, respectively, during the whole duration of the experiment, it seemed possible

that this pharmacodynamic activity of iproniazid could have masked any possible opposite effects of a MAO inhibition This conception is corroborated by the fact that some of the authors mentioned found a certain depression of the effects of catecholamines shortly after the injection of iproniazid (BALZER & HOLTZ, KAMIJO *et al*, SCHMITT & GONNARD, KOBINGER & FRIIS) as well as by some experimental findings of CHESSIN *et al* (1957) in dogs and cats pretreated with iproniazid

The work of HESS *et al* (1958) on the metabolism of iproniazid has shown this compound *to be nearly completely metabolized within* 24 hours, whereas the inhibition of the enzyme remained complete over a period of 5 to 6 days This observation makes it possible to investigate the effects of MAO inhibition without any interfering pharmacodynamic effects of the inhibitor In the experiments described below the effect of MAO inhibition, induced by pretreatment with iproniazid the day before the experiments, on the sensitivity of the nictitating membrane of the cat to noradrenaline was studied in order to elucidate any possible role of the enzyme in the inactivation of catecholamines at the receptor

Methods

The experiments were performed on 25 cats, in most of which one nictitating membrane had been denervated by exstirpation of the *ganglion cervicale superius* 8 or 9 days before the experiment One group of 10 cats remained without pretreatment as controls, 10 other cats received an i p injection of 50 mg/kg iproniazid 20 to 24 hours before they were anaesthetized for the experiment, and a third group of 5 cats was pretreated in the same manner with an i p injection of 40–50 mg/kg isoniazid

The cats were anaesthetized with pentobarbital (40 mg/kg i p) After preparation of the neck for artificial respiration the pentobarbital dose was raised to 70–100 mg/kg and gave complete respiratory arrest and muscular relaxation Cats so deeply anaesthetized behave like decerebrate preparations Both *nervi vagi* were cut in the neck, and on the side not denervated the nictitating membrane was decentralized by cutting the *n sympathicus* The arterial blood pressure was recorded in one femo...
kymograph After the cat had been fixe...
branes were connected to a lever loa...
magnification of the contractions of th...
done into one *v femoralis*

In all experiments a dose response curve was first established for the react...
both ...
8 µg
mine
0 25

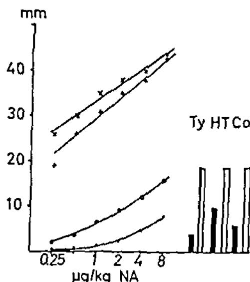


Fig 1 Influence of pretreatment with ipronia
of cats to noradrenaline (NA), tyramine (Ty)
Ordinate height of the contraction on the ky
adrenaline, right side contractions to tyran
µg/kg) and cocaine (1 mg/kg)
Left side ●——● decentralized organ controls, ○——○ decentralized organ ipromazid pretreated, +——+ denervated organ controls ×——× denervated *organ, iproniazid petreated*
Right side reactions of the decentralized nictitating membrane in controls (black bars) and after pretreatment with iproniazid (open bars)

Under *results* the contractions of the nictitating membranes are given in mm excursion of the lever on the kymograph

The significance of the differences between the pretreated groups and the controls was tested by analysis of variance and by the ranking method of Wilcoxon (*1947*)

Results.

The results of our studies are summarized in fig 1 and table 1

The decentralized nictitating membranes of cats pretreated with iproniazid were more sensitive to noradrenaline than those of the controls The difference became statistically significant at a dose of 0 5 µg/kg, to which the average control response amounted to only 0 65 mm and remained so on the higher doses Likewise, the iproniazid-pretreated animals showed greater responses to tyramine and 5-hydroxytryptamine, both considered better substrates of monoamine oxidase than noradrenaline Finally, the reaction of the decentralized nictitating membrane to cocaine was significantly greater in the iproniazid-pretreated group

Cats pretreated with isoniazid showed no corresponding effect, the reactions of the decentralized side even tending to be smaller than in the control group

Table I

Average responses (mm) of the decentralized nictitating membranes to noradrenaline, tyramine, 5 hydroxytryptamine and cocaine

Treatment	0.25	0.5	1	2	4	8	0.1 mg/kg tyramine	25 µg/kg 5 HT	1 mg/kg cocaine
	µg/kg noradrenaline								
Iproniazid	2	3.5	6.5	9	12	16	19	19	19
p	< 0.05	< 0.05	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.05	< 0.01
Controls	0.3	0.65	1.2	2.2	4.4	7.6	4	9.9	5.9
p	> 0.05	> 0.05	> 0.05	< 0.01	> 0.05	> 0.05	> 0.05	> 0.05	< 0.01
Isoniazid	0	0.1	0.3	1	3.1	5	0.6	5	0.6

On the other hand the reactions of the decentralized organ in the iproniazid group remained distinctly lower than those of the denervated nictitating membranes, and the sensitizing effect of cocaine to noradrenaline was also still demonstrable. The response to the two noradrenaline doses tested after cocaine was not significantly different in controls and iproniazid group, though it seemed to be slightly higher in the latter.

The denervated nictitating membranes showed an apparent trend towards slightly higher reactions in the iproniazid group also, but the difference from the control was in no instant significant.

Discussion

The results of this study show that it is possible to sensitize the nictitating membrane, and probably also other sympathetic effector organs, to the effects of noradrenaline as well as to other substrates of MAO by inhibition of this enzyme. That the contraction of the nictitating membrane to cocaine is also intensified seems not surprising, since this action is due to endogenously liberated noradrenaline (KUKOVETZ & LEMBECK 1962). However, the sensitizing effect to noradrenaline seems not to become obvious before the concentration of the inhibitor iproniazid has fallen below a level above which the compound explays pharmacodynamic effects of its own, in other words, it occurs only under the conditions of a rather 'pure' enzyme inhibition. This explains that several authors in acute experiments have failed to detect any sensitizing effect of inhibitors of MAO on the effects of catecholamines (GRIESEMER *et al* 1953, BURN *et al* 1954, BALZER & HOLTZ 1956, SCHMITT & GONNARD 1956), or, when they have found such an effect (KAMIJA *et al* 1956), thought it to be an unspecific property of hydrazides, since isoniazid also, though not an

inhibitor of MAO, had this effect for *some hours after injection* In our experiments, isoniazid had no such effect 20 to 24 hours after its injection

The sensitizing effect resulting from the inhibition of MAO was clearly less than that of cocaine and chronic denervation but a trend to higher contractions in the iproniazid pretreated group could still be shown after cocainization and in the denervated nictitating membranes

These findings indicate a different mechanism of action for the three possible ways of sensitizing the nictitating membrane to catecholamines The sensitizing effect of chronic denervation is thought to be due to an atrophy of the catecholamine tissue stores in the nictitating membrane, thus inhibiting the uptake of noradrenaline into the tissue and providing higher *concentrations at the specific receptors* (HERTTING *et al* 1961, KUKOVITZ & LEMBECK 1962), whereas cocaine prevents the inactivation of catecholamines by uptake into the tissue *stores*, thus leading to an effect similar to chronic denervation (MUSCHOLL 1960) The most probable *reason for the sensitizing effect of* MAO *inhibition shown in* our experiments seems to be an interference with the chemical metabolism of catecholamines The investigations of AXELROD *et al* (1960, 1961) leave no doubt that MAO does not play any important part in the inactivation of the bulk of injected noradrenaline, but they cannot exclude the possible importance of *the* enzyme *for the* inactivation of the *small share* reaching the specific receptors The sensitizing effect of MAO inhibition could *then be a consequence of the slower breakdown of the catecholamines* fixed to the specific receptors This concept to some extent supports the finding of JOHNSON & SELLERS (1962) that the thermogenic effect of noradrenaline could be enhanced by an inhibitor of MAO, but not by inhibition of O-methyltransferase

If our conception of the part played by MAO in the inactivation of receptor bound catecholamines is valid it could explain why the sensitizing effect of chronic denervation is usually greater than that due to cocaine Whereas cocaine only prevents the uptake of catecholamine into the tissue stores, *and has no demonstrable inhibitory effect on* MAO *in vivo*, chronic denervation not only prevents the uptake into the atrophied tissue stores, but also leads to an MAO depletion of up to 70% (BURN & ROBINSON 1952) The inhibition of the remaining MAO by iproniazid could account for the observed slightly higher contractions of the denervated nictitating membranes in cats pretreated with iproniazid (fig 1)

Summary

Some 20 to 24 hours *after the injection of iproniazid, that is*, at a time *when there exists an inhibition of monoamine* oxidase *not complicated by*

ny pharmacodynamic effects of the inhibitor, the decentralized nictitating nembranes of cats proved to be sensitized to the effects of injected nor-drenaline However, this effect was not so pronounced as the sensitiza-ion by cocaine or chronic denervation Isoniazid had no corresponding ffect The results seem to point to the responsibility of monoamine oxidase for chemical inactivation of the particular portion of catechola-nines fixed to the specific receptors

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REFERENCES

Axelrod J Biochemical factors in the activation and inactivation of drugs *Naunyn Schmiedeberg s Arch exp Path Pharmak* 1960 238 24–34

Axelrod J

Balz

Pharmak 1956 227 547 558

Burn J H & J Robinson Effect of denervation on amine oxidase in structures innervated by the sympathetic *Brit J Pharmacol* 1952, 7 304–318

Burn J H F

Che

Griesemer E C J Barsky C A Dragstedt J A Wells & E A Zeller Potentiating effect of iproniazid on the sympathom

Griesemer E C blockade b

Hertting G J uptake of catecholamines after chronic denervation of sympathetic nerves *Nature* (Lond) 1961 189 664

Hess S H Weissbach B G Redfield & S Udenfriend The between iproniazid me amine oxidase *J Phar*

Johnson G E, & E A S on the thermogenic ef 40 631 638

Kamijo K G B Koelle & H H Wagner sympath I isonic zide (IN

Kobinger W lierten *Schmied*

Krause D Beitrag zum Mechanismus der Wirkungsverstärkung des Nor Adrenalins durch Cocain *Naunyn Schmiedeberg s Arch exp Path Pharmak* 1961 241 201 202

Kukovetz, W R & F Lembeck Untersuchungen uber die adrenalinpotenzierende Wirkung von Cocain und Denervierung *Naunyn Schmiedeberg s Arch exp Path Pharmak* 1962, 242 467–479

Muscholl, E Die Hemmung der Noradrenalin Aufnahme des Gewebes durch Cocain *Naunyn Schmiedeberg s Arch exp Path Pharmak* 1960 240 8
Philpot F J The inhibition of adrenaline oxidation by local anaesthetics *J Physiol* (Lond) 1940 97, 301–307
Schmitt H , & P Gonnard Modifications par un inhibiteur de l aminoxy dase l iproniazide des effets de quelques amines sympathicomimetiques sur la membrane nictitante du chat *Arch int Pharmacodyn* 1956 109 74–83
Wilcoxon, F Probability tables for individual comparisons by ranking methods *Biometrics* 1947, 3 119 122

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From the Department of Pharmacology, University of Copenhagen
(Professor Knud O Møller, M D)

Effects of Cholinergic Agents on Intestinal Absorption of Magnesium by Rabbits

By

Jens Jensen-Holm

(Received April 16 1963)

Oral administration of magnesium sulphate will, even after large doses, generally lead only to minor rises in plasma levels of magnesium The causes of this are said to be that 1) Mg^{++} is absorbed to a fairly small extent only, 2) the metal is rapidly distributed over the liquid phase, but less so intracellularly (ENGBÆK 1952) and 3) that renal excretion proceeds fairly quickly, depending on the rate of urine flow and the plasma level The rate of intestinal absorption increases on administration of the salt at a high concentration (TERKILDSEN 1950, SCHATTMANN 1951, JENSEN-HOLM 1962) After rectal injection into rabbits the absorption of magnesium, as estimated by the rise in plasma level, will proceed up to 20 times more rapidly if the solution of magnesium sulphate is made hypertonic with glycerol, glucose or urea or if the intestine has been pretreated with a hypertonic solution of one of these substances (JENSEN HOLM 1962) At a molar concentration of not less than 0 9 (excluding $MgSO_4$) in the enema given, complete magnesium anaesthesia developed in all the animals, with plasma magnesium levels ranging from 160 to 195 µg/ml Several animals died A similar effect has not been obtainable by oral administration of corresponding hypertonic solutions (JENSEN HOLM 1962a, unpublished)

TERKILDSEN (1952a) has shown that intramuscular injection of physostigmine neostigmine or acetylcholine into rabbits caused minor rises in plasma magnesium This effect was believed to be due to loss of magnesium from the intracellular environment

The object of the study here recorded was to investigate whether cholinergic agents, administered by mouth together with $MgSO_4$, alter the rate of intestinal absorption of magnesium Such a possible relation is of importance in connection with the question whether use of magnesium sulphate as a saline evacuant is suitable in the treatment of poisoning

due to oral intake of certain poisons. TERKILDSEN (1950, 1952), however, found no unequivocally increased absorption of magnesium after oral administration of $MgSO_4$ to soporific-poisoned patients

General Methods

Magnesium was determined by the Titan Yellow method (KOLTHOFF 1927; ANDREASEN 1957), modified so as to be more sensitive (see JENSEN-HOLM 1962). The standard curve is slightly S-shaped. All the results of analyses have been corrected accordingly. It has been shown that none of the compounds used interfered with the results of the analyses.

Compounds

$MgSO_4$, 7 H_2O (Ph D)
Paroxan*) = paraoxon, E 600 (0 0 -diethyl-0-p-nitrophenylphosphate)
Neostigmine bromide (Ph D)
Atropine sulphate (Ph D)
Carbacholum (WHO), carbacholine chloride (Ph D)
Acetylcholine iodide (Hoffman-La Roche and Co, Ltd), symbol used ACh I
Activated charcoal (USP), granulatum carbonis (Ph D)

Experimental Animals.

Coloured male rabbits were employed They weighed about 2 7 kg, with an average of 2 70 ± 0 09 (s e m)

Oral administration was performed after introducing the stomach tube The same concentration of magnesium sulphate in water (2 5 g and distilled water to 20 ml) was used in all the experiments, and except in one experiment also the same amount per kg body weight (1 10 g, corresponding to 108 5 mg magnesium) The animals used had all fasted since the preceding afternoon (for about 16 hours) Nevertheless, on autopsy of the animals that died, most of the stomachs were found to be partly filled with food residues

Blood samples were withdrawn from the marginal ear vein after this had been opened with a phlebotome The blood was drawn into a pipette washed through with a 0 1 % solution of heparin in saline The magnesium was determined in both erythrocytes and plasma Only the results of the latter are given in this paper The erythrocyte concentrations followed approximately the plasma concentrations

The initial values for the concentrations of magnesium in plasma and erythrocytes before introduction of $MgSO_4$ were found to average (71 experiments):

Plasma: 18 76 ± 0 58 µg/ml (s e m)
Erythr.: 73.80 ± 1.54 µg/ml (s e m.)

*) The paroxan used, which is chemically pure (about 99 %), was kindly placed at our disposal by Farbenfabriken Bayer, Leverkusen, Germany, through Professor W Wirth

Experimental Groups

1)
2)
3)

–	5	–					
–	0	–	–	–	(6	–	)
–	55	–	after	–	(1	–	)
–	94	–	–	–	(1	–	)
–	125	–	–	–	(1	–	)

4) $MgSO_4$ + paroxan + activated charcoal (5 experiments)
   $MgSO_4$ + paroxan + activated charcoal + ACh I (2 experiments)
5) $MgSO_4$ + paroxan + carbachole (3 experiments)
   $MgSO_4$ + paroxan + atropine (4 experiments, only 2 shown) (atropine given parenterally)
6) Paroxan + activated charcoal + ACh. I, but *no* $MgSO_4$ (1 experiment)
7) $MgSO_4$ orally Paroxan subcutaneously (5 experiments 2 shown)

Magnesium sulphate was given by mouth in all the experiments In group 5 atropine was given parenterally, and in group 7 paroxan subcutaneously

Results

1) *Control Group*

Eight rabbits received equal amounts of $MgSO_4$ per kg, given at the same concentration The results are set out in table I, in which are recorded the average plasma levels of magnesium and the standard error of the mean (s e m) The plasma level rose most rapidly during the first hour and thus remained approximately constant

In one experiment about twice as much magnesium sulphate was given, but at the same concentration as above It is seen from table 1 that here also the plasma level seemed to rise at the fastest rate within the first hour The total plasma concentration obtained was twice as high as after administering half the amount Maximal concentration seemed not to be reached within 2½ hours

Table I

Concentration of magnesium in plasma after oral $MgSO_4$ in untreated rabbits (controls) after 1 10 g/kg (8 animals) and after 2 13 g/kg (one animal)

The doses of $MgSO_4$ were given at the same concentration (2.5 g $MgSO_4$ dissolved to 20 ml destilled water) after previous fasting for about 16 hours

Time in min		0	20	40	60	90	120	150
1 10 g kg	number	8	8	8	7	7	5	2
	μg ml	18 3	23 9	29 8	32 8	33 3	35 0	36 3
	± s e m *)	1 1	1 1	1 8	2 1	1 6	2 0	–
2 13 g kg	μg ml	21 6	29 6	36 5	45 7	49 0	53 9	57 1

*) Standard error of the mean

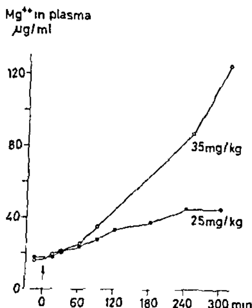


Fig 1 Neostigmine and $MgSO_4$
Abscissa Time in minutes after oral $MgSO_4$ and neostigmine bromide
Ordinate Concentration of plasma magnesium in µg/ml
Doses of neostigmine in mg/kg as indicated After 35 mg/kg a marked increase in the plasma magnesium concentration This rabbit developed symptoms of magnesium anaesthesia and died of a combinated neostigmine and magnesium poisoning

2) *$MgSO_4$ + Neostigmine by Mouth*

Fig 1 illustrates the results of two experiments with 25 and 35 mg neostigmine bromide per kg, dissolved in $MgSO_4$ solution After 25 mg/kg a slightly accelerated absorption of magnesium was observed After 35 mg/kg a pronounced rise was noticed, to about 125 µg per ml of plasma, and the animal displayed signs of complete magnesium anaesthesia The animal must be assumed to have died of combined magnesium and neostigmine poisoning

3) *$MgSO_4$ + Paroxan by Mouth*

a) *Paroxan administered 20 minutes before $MgSO_4$* Four experiments were conducted with the doses 10 1, 9 9, 7 6 or 5 4 mg/kg In the first, illustrated in fig 2, D, a pronounced rise was seen, from an initial level of 24 µg/ml to about 170 µg/ml, within no more than 9 min (after which the animal died) It developed a complete, reflexless anaesthesia, with a terminal cessation of respiration Just before the anaesthesia set in, a general restlessness of a few minutes' duration was noticed True signs of paroxan poisoning were never observed In the three other animals given smaller doses of paroxan, a markedly accelerated though more moderate absorption of magnesium was found These animals survived the experi

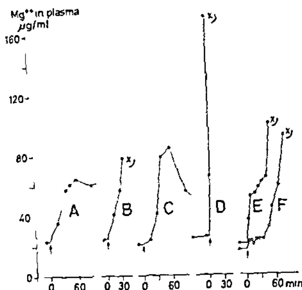


Fig 2

Abscissa Time in minutes after administration of oral $MgSO_4$
Ordinate Concentration of magnesium in plasma (µg/ml)
The figure shows 6 examples of elevated absorption of magnesium after oral $MgSO_4$ and cholinergic agents
Curve A Paroxan 9 2 mg kg Survived
Curve B Paroxan 44 2 mg + charcoal 55 mg/kg Died
Curve C Paroxan 43 3 mg + charcoal 87 mg + ACh 1 43 3 mg/kg Survived
Curve D Paroxan 10 1 mg/kg given oral 20 minutes before oral $MgSO_4$ Died of magnesium poisoning 9 minutes later
Curve E Paroxan 10 9 mg kg given oral 5 minutes before $MgSO_4$
Curve F Paroxan 10 0 mg/kg given as mentioned above The last two animals died of paroxan poisoning The magnesium plasma concentrations were markedly increased

*) The animal died at this point, the final determination was on blood taken by heart puncture

ments Results for them are not included in fig 2, but only in the survey chart, fig 4, to which reference should be made

b) *Paroxan administered 5 minutes before $MgSO_4$* Five experiments were conducted with doses of paroxan 15 7, 11 3, 10 9, 10 0 or 9 1 mg/kg After the first two doses, the animals died 1 and 2 min, respectively, after the administration of $MgSO_4$ In spite of the short interval, a doubling of the plasma level of magnesium was reached in both After 10 9 and 10 0 mg/kg (see fig 2 E and F) the absorption of magnesium rose to 98 and 91 µg/ml, respectively These animals died of paroxan poisoning before a genuine magnesium poisoning had developed After 9 1 mg/kg, a moderate rise was obtained (shown in the survey chart, fig. 4) before death occurred

c) *Paroxan administered simultaneously with $MgSO_4$* Six experiments were conducted with doses of paroxan 42 9, 17 6, 14 9, 13 7, 9 2 or 2 3 mg/kg An accelerated absorption of magnesium was seen in all the experiments (see fig 4, solid curve) The first three doses resulted in death 13, 13 and 20 min, respectively, after the oral administration, the remaining animals survived the paroxan poisoning developed in all the experiments The result of the experiment with 9 2 mg/kg is shown in fig 2, A

d) *Paroxan administered 1–2 hours after $MgSO_4$* At the time 0 three rabbits were given magnesium sulphate exactly as to the control group The first animal received after 55 min a solution containing 43 mg paroxan + 43 mg ACh I + 69 mg activated charcoal The second animal received after 94 minutes 13 8 mg paroxan per kg and the third 10 0 mg/kg after 125 minutes The second administration produced in all experiments a marked, though moderate, rise in plasma magnesium The first and the last animal died Results of these experiments have not been included in this paper

4) *$MgSO_4$ Administered together with Paroxan + Activated Charcoal* (in two animals also with acetylcholine at the same dose as paroxan)

Seven experiments were conducted Their object, carried out early in the series, was to study whether simultaneous administration of activated charcoal and fairly large amounts of paroxan might show a more distinct effect on the absorption of magnesium, without the animals dying too early of paroxan poisoning The results were, however, not particularly enlightening In all the experiments the same amount of paroxan (40–45 mg/kg) was used along with various amounts of activated charcoal (from 55 to 204 mg/kg) To two animals ACh I also was given at the same doses as the paroxan Two of the seven experiments are illustrated in fig 2B and C

5) *$MgSO_4$ Administered together with Paroxan and Carbachole*

Three rabbits were given, besides paroxan, carbachole along with magnesium sulphate by mouth The doses employed are recorded in the legend to fig 3, A Addition of the carbachole caused a rapid and significantly increased absorption of magnesium As, however, the animals died early, no particularly high concentrations of plasma magnesium were obtained Carbachole by itself with $MgSO_4$ has not been tested

$MgSO_4$ + paroxan administered by mouth to atropinised animals

Atropine sulphate, 2 mg/kg, was injected subcutaneously 15 minutes before administering $MgSO_4$ + paroxan 15 min after the latter, 1 mg

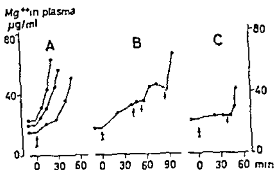


Fig 3
A Paroxan + charbachole orally
B + C Paroxan subcutaneous

Abscissa Time in minutes after oral administration of $MgSO_4$
Ordinate [illegible]
Curve A [illegible]

Lower Paroxan 7 4 mg carbachole 2 7 mg
For the last analyses blood was taken by heart puncture The animals died fairly quickly with dramatic symptoms Absorptions were rapid but the animals died before high concentrations were reached

Curve B + C Paroxan given *subcutaneously* The solution used was 2 mg/ml saline In both animals there were marked effects on the magnesium absorption ($MgSO_4$ given orally at zero point) The dose used was (*Curve B*) 0 77 mg paroxan per kg divided into three injections as indicated with arrows (*Curve C*) o 93 mg/kg at the time indicated

atropine was injected intravenously Two experiments were conducted, with 20 6 or 23 5 mg paroxan per kg Without atropine injection the doses of paroxan given would have produced a greatly increased absorption of magnesium Atropine completely abolished this action of paroxan, no appreciable absorption of magnesium having taken place during the first 70 and 30 min , respectively (see the survey chart, fig 4) In the course of the next two hours the concentration rose slowly to about 50 per cent above the initial value Atropine thus seems gradually to lose its effect Two other experiments also showed the same pronounced action of atropine (not illustrated)

6) *Paroxan + ACh I + Activated Charcoal, but No $MgSO_4$*

Only one experiment was conducted with the dose 47 3 mg paroxan + 44 4 mg ACh I + 89 mg activated charcoal per kg The plasma level of magnesium rose from an initial value of 17 5 µg/ml to 21 5 µg/ml after three hours During the same period the concentration in the erythrocytes rose from 80 to 88 µg/ml

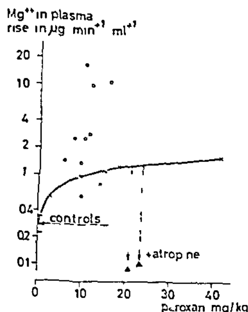


Fig 4
The effects of paroxan (mg/kg) on the absorption rate of magnesium from orally $MgSO_4$

Abscissa Dose of oral paroxan in mg/kg
Ordinate The rate of the elevation in plasma magnesium concentration measured over the first 20 minutes after administering $MgSO_4$ (or even for a shorter time, if the animal died before) The rate is expressed as µg magnesium increase per minute and per ml plasma

In absence of paroxan (at zero point) we have the absorption rates for the controls (8 animals s e m indicated)

The solid curve shows the absorption rates when paroxan was given simultaneously with $MgSO_4$ The results indicated by —○— and —●— show the absorption rates when paroxan was given before $MgSO_4$ (5 and 20 minutes before), when, as is seen the absorption rates were much greater

Administration of atropine subcutaneously (▲) markedly decreased the rate of magnesium absorption in spite of the doses of paroxan used The absorption rates were here also possibly less than in the control group

7) *$MgSO_4$ Orally and Paroxan Subcutaneously*

In five experiments paroxan was injected subcutaneously A solution was used containing 2 0 mg/ml saline Doses were given ranging from about 0 5 to 1 0 mg/kg All the animals died from 5 to 65 min after the injection A clear, though moderately, accelerated absorption of *magnesium* was observed in all the experiments The results of two of the experiments are illustrated in fig 3, B and C The highest concentration measured at death was 65 µg/ml

Discussion

It is more or less evident from these experimental results that after oral administration of $MgSO_4$ the absorption of magnesium from the alimen-

tary tract, judged by the rise in plasma magnesium concentration (and also in the erythrocytes, though not shown here) proceeds more rapidly if cholinergic agents are also administered, generally at toxic dose levels In the work described was studied the effects of acetylcholine and carbachole, neostigmine and paroxan (in other words, partly choline esters and partly one reversible and one irreversible cholinesterase inhibitor)

There were, however, certain difficulties in demonstrating with sufficient clearness the existence of an absorption mechanism cholinergically determined In particular, it was difficult to produce magnesium anaesthesia (and possibly death from magnesium intoxication) before the animals had died of poisoning by the cholinergic agent itself, but two experiments succeeded in showing this (after neostigmine, see fig 1, and after paroxan, see fig 2, D) In other experiments in which the conditions were varied, e g in several of them by adding activated charcoal to increase the amount of paroxan, a markedly accelerated absorption of magnesium was certainly found, but without such high concentrations being reached that magnesium anaesthesia developed This was due either to the animals having died earlier or to the effect being too weak or ceasing too quickly, owing to an insufficient dose of paroxan The survey given in fig 4 of all the experiments conducted with $MgSO_4$ plus paroxan shows, however, that the absorption rate of magnesium from the alimentary tract was clearly higher in the experimental group than in the control group (magnesium sulphate alone) The solid curve illustrates the results after administering paroxan along with $MgSO_4$ The effect is seen to increase with rising doses of paroxan The larger the dose, the sooner did the animals generally die of paroxan poisoning If paroxan was given 5 or 20 minutes *before* $MgSO_4$ the absorption rate rose considerably (perhaps owing to absorption from the stomach) It is also shown in fig 4 that pretreatment with parenteral atropine completely abolished the effect of even large doses of paroxan Absorption may possibly have been reduced to values lower than those obtained with magnesium sulphate alone (the control group) the absorption rate being no more than one third of that in the control group

Carbachole, given by mouth, intensifies the effect of paroxan on the absorption of magnesium Acetylcholine, likewise by mouth, has no such effect Unlike carbachole, however, it does not reduce the lethal dose of paroxan It is therefore reasonable to suppose that an effect on the absorption of magnesium must be conditioned by some absorption of the cholinergic agent The physiological inhibition of magnesium absorption is therefore unlikely to take place on the surface of the gastric or intestinal epithelium but probably in deeper structures

Subcutaneous injection of paroxan (see fig 3), like oral administration, stimulated the absorption of magnesium Hence, to produce an effect the cholinergic agent does not need to be present in the alimentary tract, but, as has been stated above, absorption of the agent must have taken place

The effect of the cholinergic agents on the absorption of magnesium could be due to their causing a prompt spread of the magnesium sulphate containing fluid over a larger absorbing surface than in the absence of cholinergic agents However, the fact that administration of twice as much magnesium sulphate, but at the same concentration, causes only a slight rise in absorption argues against this explanation The objection may also be raised that paroxan generally acts instantaneously and rapidly on the absorption of magnesium Further, the fact that paroxan administered a few minutes before magnesium sulphate accelerates absorption far more than when simultaneously administered hardly fits the primary hypothesis of a rapidly increasing absorbing surface

The possibility of reduced renal excretion of magnesium in poisoning with cholinergic agents must also be considered In the experiment illustrated in fig 2, D, in which the plasma magnesium level rose from 24 to 170 µg/ml during no more than 9 minutes, even cessation of renal excretion of magnesium would hardly have influenced the result

Paroxan administered together with acetylcholine and activated charcoal, but without magnesium sulphate, caused only slight rises in magnesium plasma and erythrocyte concentrations The rises noted in the remaining experiments must therefore be almost exclusively due to the administered magnesium sulphate

Thus, there can hardly be any doubt that *the accelerated, sometimes greatly accelerated, absorption of magnesium from the alimentary tract is indeed cholinergically determined and can be produced by paroxan for instance This action can be intensified by carbachole and abolished by atropine* Such a mechanism of action seems not to have been described before

It has been shown previously (Jensen Holm 1962) that absorption of magnesium can be greatly accelerated by rectal injection of magnesium sulphate, provided the injected solution has been rendered hypertonic with different substances (glycerol, glucose, or urea) or provided the colon has been pretreated with a hypertonic solution Such an effect has not been procurable orally by similar administration despite use of a highly hypertonic solution (Jensen Holm 1962a) We do not know for certain whether or not this is due to the mucous membranes of the proximal alimentary tract being physiologically so resistant to the action of a hypertonic solutions as to preserve its function unchanged (cf, however, G. T. G 1961)

Perspective

The results of the study reported here provide evidence that cholinergic agents have a pronounced accelerating effect on the absorption of magnesium administered by mouth as magnesium sulphate In another study (JENSEN HOLM 1963) it has been shown, likewise in experiments on rabbits, that acute arsenic poisoning, caused by sodium arsenite, causes increased absorption of magnesium (possibly through a mucosal lesion) Hypertonic solutions can act on the colon, but not on the proximal portion of the alimentary tract, by markedly accelerating the absorption of magnesium Administration of $MgSO_4$ solutions at high concentrations (without the dose being increased) likewise accelerates the absorption of magnesium

The observations reported above seem to justify the conclusion that we in fact know little about how magnesium is handled in the alimentary tract We must therefore *advise against using $MgSO_4$ as a saline evacuant*, especially in the treatment of poisoning (which is, moreover, often associated with impaired renal function)

Summary.

1) Neostigmine and paroxan given by mouth to rabbits accelerated the absorption by the alimentary tract of the magnesium fraction of orally administered magnesium sulphate

2) This action of paroxan could be intensified by carbachole, but not by acetylcholine, given by mouth

3) Atropine, given parenterally, completely abolished the effect of paroxan on the absorption of magnesium Atropine possibly also inhibited the rate of physiological absorption

4) The action of paroxan depended on the dose The action was appreciably increased by giving paroxan 5 or 20 min before $MgSO_4$ Paroxan given 1 or 2 hours after, on the other hand, caused only slight rises in plasma magnesium

5) Paroxan injected subcutaneously accelerated the absorption of magnesium from the alimentary tract

6) *It is emphasized* that our knowledge about the absorption of magnesium from the alimentary tract is slight We therefore advise against $MgSO_4$ in the treatment of poisoning

Acknowledgement

The author is indebted to Miss Karin Dyhrfjeld for valuable technical assistance

REFERENCES

Andreasen, E On the determination of magnesium in serum and urine by The Titan Yellow method *Scand J Clin & Lab Invest* 1957, 9, 138-143

Engbaek, L The pharmacological actions of magnesium ions with particular reference to the neuromuscular and the cardiovascular system *Pharmacol Rev* 1952, 4, 396-414

Fawcett, D W & J P Gens Magnesium poisoning following an enema of epsom salt solution *J A M A* 1943, 123, 1028-1029

Jensen Holm, J Effects of different hypertonic solutions on the absorption of magnesium from the rabbit colon *Arch int Pharmacodyn* 1962, 140 105-114

Jensen-Holm, J Unpublished, 1962a

Jensen-Holm, J Effect of sodium arsenite intoxication on the intestinal absorption of magnesium in rabbits *Acta pharmacol et toxicol* 1963, 20, 109-112

Kolthoff, J M Eine neue spezifische Farbreaktion auf Magnesium und eine einfache kolorimetrische Methode zur quantitative Bestimmung von Spuren dieses Elements *Biochem Z* 1927, 185, 344-348

Schattmann, K Beitrag zur Therapie der Leber- und Gallenweg Erkrankungen mit Magnesiumsulfat *Artzl Wschnschr* 1951, 6, 340-344

Terkildsen, T C Nogle undersøgelser over serum magnium Thesis Copenhagen 1950

Terkildsen, T C Serum magnesium, in acute barbituric acid poisonings treated with oral administration of magnesium sulphate *Acta pharmacol et toxicol* 1952, 8, 379-384

Terkildsen, T C The effect of physostigmine, neostigmine and acetylcholine on serum magnesium concentration *Acta pharmacol et toxicol* 1952 a, 8, 385-390

Ostling, G Stratification of absorbed solutions in mesenterial veins *Acta physiol scand* 1961, 52, 254-266

Acta pharmacol et toxicol 1963, 20, 109–112

From the Department of Pharmacology University of Copenhagen
(Professor Knud O Moller, M D)

Effects of Sodium Arsenite on the Intestinal Absorption of Magnesium in Rabbits

By

Jens Jensen-Holm

(Received April 16 1963)

It has been shown in a previous study (JENSEN HOLM 1963) that cholinergic agents, specially in toxic doses, given orally or parenterally, can accelerate the absorption of magnesium from the intestinal tract An underlying cholinergic mechanism was revealed It was also pointed out that the normal physiological inhibition of the absorption of magnesium from the alimentary tract can hardly be localised in the mucosal surface itself, but must be found in deeper structures of the wall Hypertonic solutions can greatly accelerate the absorption of simultaneously or subsequently administered magnesium sulphate from the colon (JENSEN-HOLM 1962), but not from the proximal portion of the alimentary tract (JENSEN HOLM 1962a)

To throw further light on the intestinal absorption of magnesium, with special reference to poisoning we have chosen to study the effect of arsenic (sodium arsenite) on the absorption of magnesium Such a study is of particular interest, since it is stated in many pharmacological and medical text books that introduction of large amounts of a [illegible]

[illegible] presented here that the absorption of magnesium is accelerated in arsenite poisoning

Methods

Magnesium was determined in plasma and erythrocytes on the lines indicated previously for *blood sampling* and *analytical technique* (JENSEN-HOLM 1962, 1963)

Male rabbits (coloured) weighing about 2 8 kg were used Magnesium sulphate and sodium arsenite were administered by stomach tube

Compounds.

$MgSO_4$, 7 H_2O (Ph D)
Sodium arsenite (Kahlbaum, Berlin)

Magnesium determinations Several analyses showed that sodium arsenite did not interfere with the magnesium determination, as high a concentration of sodium arsenite as 10 mg/ml altered neither the blank nor the extinction measured in the presence of 100 µg Mg^{++} per ml The observed rises in plasma magnesium level were therefore solely due to an increased amount of magnesium

Results.

The results are set out in fig 1, which illustrates partly the absorption in eight rabbits (controls, average curve) and partly the absorption after oral administration of $MgSO_4$ as well as sodium arsenite in aqueous solution Curves have been plotted to show the course in three rabbits

1) 110 mg Na_3AsO_3 per kg at the time 0 About one hour later $MgSO_4$ (1.1 g/kg and 12 5 per cent w/v) was administered by mouth The animal

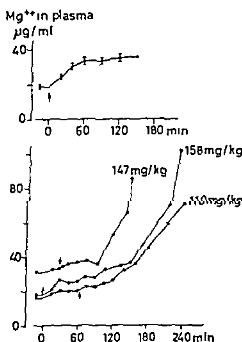


Fig 1

Abscissa ... tes after orally administered sodium arsenite
Ordinate ... are given
Upper curve ... ndicated)
Lower curves ... explana

died 4 hours after the administration of Na_3AsO_3. The plasma magnesium level had then risen from 15.9 to 70.5 µg/ml.

2) 147 mg Na_3AsO_3 per kg at time 0. 30 min. later $MgSO_4$ was administered as under (1). The animal died 145 min. after administering the sodium arsenite. The plasma magnesium level had then risen from 30.9 to 85.4 µg/ml.

3) 158 mg Na_3AsO_3 per kg at time 0 simultaneously with administration of $MgSO_4$ (in the same solution). The animal died after 231 min. The plasma magnesium level had then risen from 17.5 to 101.5 µg/ml.

For comparison, the course is indicated in fig. 1 for eight rabbits given $MgSO_4$ alone. The rise in plasma magnesium averaged 18 µg (from mean initial level of 18 µg/ml to a maximum of 36 µg/ml).

Discussion

It has been shown in three experiments on rabbits that the absorption of magnesium from orally administered $MgSO_4$ is greatly accelerated in the presence of acute fatal arsenic poisoning caused by sodium arsenite. In the control animals the plasma magnesium level was found to rise on the average by 18 µg/ml. In the three arsenite poisoned animals, up to the moment of death the level rose, by 54.6, 54.5, and 84.0 µg/ml, in other words, a rise of three to five times that observed in the controls. In two further experiments $MgSO_4$ was administered after the sodium arsenite: both animals died, 5 and 7 min. later. In one a pronounced rise in plasma magnesium was seen until death occurred, in the other relatively little effect was noticed.

It seems clear that the alterations in the mucous membranes of the alimentary tract (and capillaries) due to arsenite poisoning result in an appreciably increased absorption rate of magnesium after oral administration of magnesium sulphate dissolved in water. There is reason to suppose, however, that renal excretion of magnesium is impaired at the same time. It should be noted that the time at which the absorption curves leave the course of the control curve coincides with that of the first intestinal sounds: a few minutes later occurs the first pasty faecal discharge. That the plasma magnesium level does not reach still higher values is probably due to the fact that the administered magnesium sulphate is discharged with the faeces before appreciable absorption has taken place. The excretion of magnesium sulphate with the faeces must be assumed to proceed at a faster rate in arsenite poisoning than after a pure saline action.

Owing to the observations recorded above, it is considered inadvisable to recommend the use of $MgSO_4$ as part of the treatment of acute poisoning by oral arsenic. (Cf. *discussion*, JENSEN HOLM 1963).

Summary.

It has been shown that in rabbits acute arsenic poisoning caused by sodium arsenite raises the absorption rate of magnesium after oral administration of magnesium sulphate. The observed rise in plasma magnesium was 3 to 5 times higher than observed in controls

REFERENCES

Jensen-Holm, J. Effects of different hypertonic solutions on the absorption of magnesium from the rabbit colon *Arch int Pharmacodyn* 1962, 140, 105–114

Jensen-Holm, J Unpublished 1962a

Jensen-Holm, J Effects of cholinergic agents on the intestinal absorption of magnesium in rabbits *Acta pharmacol et toxicol* 1963, 20, 97–108

Acta pharmacol et toxicol 1963, 20, 113–114

Names approved by The Nordic Pharmacopoeia Council[1]) (NFN Names).

(Received May 18 1963)

The Nordic Pharmacopoeia Council announces its approval of NFN[2]) names for the substances in the list below These names may be used should the substances be included in the forthcoming Nordic Pharmacopoeia, in one or more of the Nordic national pharmacopoeias and official formularies or in addenda to any of these

The substances for which these NFN names have been approved are identified by giving their chemical names first, secondly the most frequently used non proprietary names, including the International Non Proprietary Names approved by WHO, and thirdly a selection of the best known registered trade names and other names of pharmaceutical specialities (preceded by the letter ®) applying either to the substance itself or to salts of it or to preparations of which it is the active principle This information is given in a similar manner to that in the Council's publication "NFN-navne"[3]) both in volume of information and in use of abbreviations

Names approved in 1962[4])

NFN name	*Other Names*
Acephenazinum maleate acephenazini *maleas*	2 Acetyl 10-[3 (4-(2 hydroxyethyl)piperazinyl (1)) propyl] phenthiazine pINN Acetophenazinum ® Tindal (Psycho-sedative)

[1]) The General Secretary Fack Stockholm 60 Sweden

[2]) NFN Abbreviation of the Nordic name (" Nordiska Farmakopé nämnden ") for the Nordic Pharmacopoeia Council

[3]) NFN navne 2nd edition Copenhagen Helsingfors, Reykjavik, Oslo and Stockholm 1958 with Addendum 1961

[4]) Names approved in 1961 see this journal vol 19 1–15 1962.

NFN-name	*Other Names*
Acidum xenyhexenicum (pINN)	1-(Biphenylyl-(4)) pentene (3)-carboxylic acid (1) ®: Desenovis (Against hypercholesterolaemia)
Clochinatum (BAN, pINN)	7-Chloro-4-(4-diethylammonio-1-methylbutylamino) quinolinium-bis(8-hydroxy-7-iodoquinoline sulfonate-(5)) pINN: Cloquinatum (BAN) ® Resotren (Chemotherapeutic)
Demecastigminum bromide: demecastigmini bromidum	N,N'-Decamethylenbis(trimethyl(3-methylcarbamoyloxyphenyl)ammoniumhydroxide) rINN: Demecarii bromidum (BAN, NND) ® Humorsol, tosmilen (Anticholinergic)
Detrothyroninum Sodium salt: detrothyronin-natrium (pINN)	*D*-3-[4-(4-Hydroxy-3-iodophenoxy)-3,5-diiodophenyl] alanine (Against hypercholesterolaemia)
Methoxifluranum (pINN)	2,2-Dichloro-1,1-difluoro-1-methoxyethane pINN Methoxyfluranum ® Penthrane (Anaesthetic)
Tolclotidum	5-Chloro-2,4-disulfamoyltoluene pINN Disulfamidum BAN. Disulphamide ® Disamide (Diuretic)
Xenygloxalum (pINN)	4,4-Diglyoxyloylbiphenyl ® Xenalvis (Chemotherapeutic)

Acta pharmacol et toxicol 1963, 20, 115–120

From the Research Division Wyeth Laboratories Inc
Philadelphia 1, Pennsylvania

Effects of the Potassium and Magnesium Salts of Aspartic Acid on Ammonia Intoxication in the Rat

By

Harry Rosen, Albert Blumenthal and Albert Consalvi

(Received June 24 1963)

The production and alleviation of ammonia intoxication in experimental animals has received attention in recent years Much of the work has been undertaken in the hope that it would establish a relationship between elevated blood ammonia concentration and the coma that commonly occurs during hepatic failure Only a few of the many published articles will be cited here

It has been shown (GULLINO *et al* 1956, GREENSTEIN *et al* 1956, DU RUISSEAU *et al* WINITZ *et al* 1956) that increased blood ammonia levels and death produced by some of the essential amino acids or ammonium acetate in the rat could be relieved by L arginine or other related compounds INGLE & WILLIAMS – ASHMAN (1962) studied ammonium acetate intoxication in the normal and in the partially hepatectomized rat They found that partial hepatectomy lowered the tolerance of rats for dietary ammonium acetate and that L arginine afforded protection against ammonium acetate given intraperitoneally to either normal or partially hepatectomized animals

Using unanaesthetized dogs HANDFORD (1959) concluded that disodium α ketoglutarate, L arginine and monosodium glutamate were ineffectual in combatting ammonia intoxication On the other hand, BARAK *et al* (1962) found that monosodium glutamate and arginine glutamate reduced ammonia intoxication in the *Eck fistula dog* *In other studies* LABORIT (1958) reported that potassium and magnesium aspartates effectively antagonized *increased* ammonia levels during haemorrhagic shock in rabbits and also *prevented* the rise of ammonia levels in rats during exercise He stated also *that rabbits* were similarly protected against ammonium chlor de –

sit

me [illegible] ammonium acetate

Methods and Materials

Male rats ranging in weight from 182 to 328 grams, mostly in the range of 240 to 280 grams, were used in this study All animals were fasted for 24 hours before the experiment

In a first series, solutions of the test agents in water (15 ml/kg) were administered intraperitoneally, one hour later each rat was challenged by the same route with 800 mg/kg of ammonium acetate in 25 ml water Previous experiments had established this as an LD70 Symptoms were observed, with death as the end point

The same schedule was followed in a second series, except that 15 minutes after ammonia challenge the rats were beheaded and the ammonia levels were determined from the collected blood by the method of NATHAN & RODKEY (1957)

The materials tested were

DL-Potassium aspartate	$\frac{1}{2}H_2O$ – mol wt – 180 2
L-Potassium aspartate	$2H_2O$ – - - – 207 2
Potassium chloride	– - - – 74 6
DL-Sodium aspartate	H_2O – - - – 173 1
DL-Magnesium aspartate	$4H_2O$ – - - – 360 5
L-Magnesium aspartate	$4H_2O$ – - - – 360 5
Magnesium chloride	$6H_2O$ – - - – 203 3
L-Arginine hydrochloride	– - - – 210 7
L-Monosodium glutamate	– - - – 169 1

Results and Discussion

Table 1 lists the protection against ammonia deaths obtained with the individual compounds used All doses of potassium and sodium salts on the horizontal lines of this table are equimolar, likewise all doses of the magnesium salts on the horizontal lines are equimolar Values for % protection were corrected for non-mortality by the method of FINNEY (1947)

Racemic potassium and magnesium aspartates each protected rats against death from ammonium acetate, the potassium salt was more effective on a weight for weight basis This protection was not due to the potassium and magnesium ions alone, since the administration of potassium and magnesium chlorides in amounts giving the same cation content as the aspartate salts showed at best protection that was only erratic and not equal to that of the aspartate salts This lack of protection from death by the chlorides was not due to inherent toxicity, acute toxicity determinations with both salts produced no mortality at the dose levels used in these experiments Similarly, the aspartate moiety did not in itself account for all the protection Sodium aspartate, though giving some protection, was not as effective as potassium aspartate It is therefore held that the combination of cation and anion is important

In these experiments L arginine hydrochloride and L-monosodium glutamate also protected against ammonium acetate deaths This confirms the results obtained by GREENSTEIN *et al* (1956) on rats

Potassium and magnesium aspartates, when tested individually at 358 mg/kg, gave only minimal protection When mixed together they gave a degree of protection in excess of the added values (Table 2) This indicates that these materials potentiate each other It is again apparent that *the potassium, magnesium* and aspartate ions do not separately provide maximum protection, all components must be present at the same time Neither sodium and magnesium aspartates nor potassium and magnesium chlorides protect as well as does a mixture of potassium and magnesium aspartates It was apparent also that an equal mixture of the *two produced the best effect this was demonstrated* by holding either component constant at 358 mg/kg and reducing the quantity of the other, protection was greatly reduced

The results shown in Tables 1 and 2 also indicate that spatial configuration plays a role in the protection afforded by potassium and magnesium aspartates *Thus, L-potassium aspartate was as active as the racemic* form, an indication that the D form is of like potency However, the L magnesium aspartate showed small protection at the highest dose used, suggesting that the D-component of the DL mixture accounted for most of the activity This was further shown by the results obtained with an equal weight mixture of the L-forms of potassium and magnesium aspartates Dose for dose, protection was not nearly as good as with the racemic mixture, in fact the results with the L-mixture were similar to those with DL-potassium aspartate alone

Table 1

Individual Components Antagonizing LD70 Ammonium Acetate[a]

DL-K Aspartate		L-K Aspartate		K Chloride		DL-NA Aspartate		DL-Mg Aspartate		L-Mg Aspartate		Mg Chloride		L-Arginine HCl		L-Mono-Na Glutamate	
mg kg	Prot	mg kg	Prot.	mg kg	% Prot	mg kg	% Prot.	mg kg	% Prot.	mg kg	% Prot	mg kg	% Prot.	mg kg	% Prot.	mg kg	% Prot
900	100																
715	100	822	100	296	0	687	64	900	73	900	20			418	100	1006	91
568	91	653	82	235	29	546	11	715	55			403	20	209	82	834	82
506	53							568	55	568	2	320	0	104	46	671	38
451	38	519	55	187	0	433	0							52	11	447	20
358	20	412	20	148	0	344	0	451	38			254	46			336	2
								358	11	358	0	202	20				
								285				161	0				

a 16 Animals Per Dose

Methods and Materials

Male rats ranging in weight from 182 to 328 grams, mostly in the range of 240 to 280 grams, were used in this study All animals were fasted for 24 hours before the experiment

In a first series, solutions of the test agents in water (15 ml/kg) were administered intraperitoneally, one hour later each rat was challenged by the same route with 800 mg/kg of ammonium acetate in 25 ml water Previous experiments had established this as an LD70 Symptoms were observed, with death as the end point

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Potassium chloride	– " "	– 74 6
DL-Sodium aspartate	H_2O – " "	– 173 1
DL-Magnesium aspartate	$4H_2O$ – " "	– 360 5
L-Magnesium aspartate	$4H_2O$ – " "	– 360 5
Magnesium chloride	$6H_2O$ – " "	– 203 3
L-Arginine hydrochloride	– " "	– 210 7
L-Monosodium glutamate	– "	– 169 1

Results and Discussion

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unprotected rats Additional tests were made with two doses not shown in the table A lower dose mixture of 80 mg/kg of each component gave no significant reduction in blood ammonia At the other extreme, a dose of 715 mg/kg of each gave no greater reduction than did 358 mg/kg

Summary

Death in rats from ammonium acetate intoxication was prevented by DL-potassium and magnesium aspartates, either alone or together Used singly, potassium aspartate was more effective than magnesium aspartate on a weight for weight basis Relatively ineffective doses of each, when used together, afforded a high degree of protection Equal weight mixtures were found to be superior to unequal weight mixtures The aspartate moiety was shown to be necessary as well as the potassium and magnesium cations The activity of potassium aspartate is found in both the D and L isomers, whereas the D isomer accounts for most of the magnesium aspartate activity Pre treatment with potassium and magnesium aspartates significantly reduced the hyperammonaemia produced by intraperitoneal administration of ammonium acetate

Acknowledgement

We thank George Hudyma and Calvin Triol for technical assistance and Miss Ann Gillen for the statistical evaluations

REFERENCES

Barak A J F L Humoller D J Mahler & J M Holthaus The Effect of Arginine and Glutamate on Ammonia Tolerance *Gastroenterology* 1962, 43 35-42.

du Ruisseau J P, J P Greenstein M [illegible] on the Metabol[illegible]

[illegible] Studies on the Metaboli[illegible] *in vivo* III Prevention of[illegible] Compounds *Arch B oche*[illegible]

Gullino P M [illegible]

Han[illegible]

Ingle[illegible] Toxicity of Ammonium Acetate in Rats. *Arch Pathol* 1962, 73 343-351

Laborit, H B Weber J Jouany P Niaussat & C. Baron Le Metabolisme de l ammoniaque et ses perturbations Importance therapeutique des sels de l acide aspartique *Presse Med* 1958, 66 2125-2128

Table 2

Mixtures antagonizing LD70 Ammonium Acetate

Mixture	mg/kg	n	% Protection
DL-K + Mg Aspartates	358 + 358	25	71
	285 + 285	25	66
	226 + 226	25	14
	358 + 240	25	14
	358 + 120	25	3
	240 + 358	25	9
	120 + 358	25	31
L K + Mg Aspartates	653 + 568	16	82
	519 + 451	16	73
	412 + 358	16	38
	328 + 285	16	20
	260 + 226	16	20
K + Mg Chlorides	187 + 254	16	2
	148 + 202	16	0
DL Na + Mg Aspartates	344 + 358	16	29

Table III demonstrates the reduction in blood ammonia levels caused by the aspartates used together Owing to the fact that daily variation was relatively great, each dose level was treated essentially as a separate experiment, each had its own water ammonium acetate control Ammonium acetate produced a level of ammonia many times greater than normal blood levels found in these rats All three dose mixtures shown in the table reduced blood ammonia significantly compared with those of

Table 3

Blood Ammonia

Treatment	mg/kg	n	µg NH_3/ml Blood	pα
DL K + Mg Aspartates	120 + 120	24	29 0 ± 1 38b	< 025
Water		24	34 1 ± 1 68	
DL K + Mg Aspartates	240 + 240	24	28 5 ± 2 13	< 005
Water		24	38 0 2 36	
DL K + Mg Aspartates	358 + 358	20	21 7 1 80	< 005
Water		18	33 9 2 12	
Control		30	1 45 + 0 132	

α – Analysis of Variance
b – Mean ± S E M

Acta pharmacol et toxicol 1963, 20, 121–130

From the Department of Pharmacology, University of Copenhagen
(Professor Knud O Møller, MD)

Connective Tissue Electrolytes During Acidosis and Alkalosis in Normal and Oestradiol-Treated Mice

By

Hans Langgård, Jens Jensen-Holm and Eigill Hvidberg

(Received July 13, 1963)

Earlier studies have indicated that the loose connective tissue of the skin serves as a reservoir of electrolytes

In the normal condition the connective tissue contains an excess of chloride – in itself an intriguing fact Calculations of the concentrations of sodium and chloride in plasma and connective tissue further indicate that only a part of the tissue water dissolves electrolytes (EILERS & LABOUT 1946) Under the influence of diverse stimuli, electrolytes may rapidly be mobilized from the tissue, or the tissue may deposit variable quantities of electrolytes It has been shown by HVIDBERG, JENSEN HOLM & LANGGÅRD (1963) that sodium can be *deposited* in the connective tissue without any increase of plasma concentration and on the other hand that severe hypernatraemia is not necessarily followed by an increase of the sodium concentration in the connective tissue Accordingly WINTERS *et al* (1958) have shown that the amounts of sodium in the bone were unaffected by pronounced hyponatraemia or hypernatraemia It thus appears that the amounts of water and the concentrations of the different electrolytes in the connective tissue in various circumstances can be changed independently of each other and of the plasma values

The mechanisms of this depot function are poorly understood Previous evidence has indicated that the colloids of the connective tissue ground substance especially the acid mucopolysaccharides, play an important part in that mechanism, possibly because the macromolecules of the interstitial space can act as mixed cation anion ion exchangers (*cf* USSING *et al* 1960) However, other possibilities of binding or releasing ions in the connective tissue do exist and may act simultaneously or integrally or both

Nathan, D G & F L Rodkey A Colorimetric Procedure for the Determination of Blood Ammonia *J Lab Clin Med* 1957, 49, 779-785
Winitz, M, J P du Ruisseau, M C Otey, S M Birnbaum & J P Greenstein Studies on the Metabolism of Amino Acids and Related Compounds *in vivo* V Effects of Combined Administration of Nonprotective Compounds and Subprotective Levels of L-arginine HCl on Ammonia Toxicity in Rats *Arch Biochem Biophys* 1956, 64, 368-373

MORGAN as modified by BLIX (*cf* SCHMIDT 1958) For determining hydroxiproline the method of NEUMAN & LOGAN as modified by MARTIN & AXELROD (1952), was employed Tissue and plasma Na and K were determined with a Beckman DU flame photometer and photomultiplier (HVIDBERG JENSEN HOLM & LANGGÅRD 1963) The amounts of chloride in tissue and plasma were measured by automatic potentiometric titration (Radiometers titrator) in the tissue after hydrolizing with 0 6N KOH and precipitation of the proteins by Somogyi s technique (*cf* COTLOVE 1962) The pH of whole blood was determined by Radiometers thermostatically controlled capillary glass electrode without exposure of the blood to air The pH was then determined after shaking in an equilibrating chamber (tonometer) at a CO_2 tension of 40 mm of Hg From these results (so called standard bicarbonate) and the tension of CO_2 (pCO_2) in the plasma were calculated with the aid of the Astrup nomogram as modified by SIGGAARD ANDERSEN (1962) (*cf* ASTRUP 1954 PRÆTORIUS 1954 and the survey by SIGGAARD-ANDERSEN 1963) (The last mentioned author recommends the term basosis instead of the unfortunate " alkalosis")

Results

The contents of the various constituents of the tissues are expressed in meq mg or g per 100 g of the dry fat free tissue The concentrations in the tissue water and in the plasma are expressed in meq per litre Statistical computations are based on the T-test, and the mean values are given with their standard errors (*s e m*)

Because of technical difficulties in obtaining sufficient quantities of arterial blood the effects of the acidifying and alkalising treatments have been judged from analyses carried out on venous blood To meet the objections that could be raised to this, simultaneous determinations of pH and plasma bicarbonate were carried out on venous and arterial blood from separate groups of mice The results of this experiment are given in table 1 The figures indicate that the values for arterial and

Table 1

Comparison between pH and corrected bicarbonate values in arterial and venous blood

	pH					Corrected bicarbonate of plasma in meq/l				
	arterial blood			venous blood under anaesthesia		arterial blood			venous blood under anaesthesia	
	n	mean	range	n	mean s e m	n	mean	range	n	mean s e m
Control	6	7 34	7 27–7 41	23	7 14 ± 0 015	6	19 9	17 2–21 2	23	15 7 ± 0 39
Acidotic animals	5	7.28	7 21–7 31	22	7 09 ± 0 013	5	15 7	13 1 16 8	22	12 6 ± 0 32
Alkalotic animals	5	7 41	7.36–7 52	25	7 20 ± 0 014	5	24 4	20 7 28 0	25	18 6 ± 0.46

Our study was undertaken to determine whether experimentally induced non respiratory acidosis and alkalosis in the blood, which are presumably accompanied by concomitant changes in the pH of the extracellular tissue buffer systems, cause a shift of electrolytes between plasma water and connective tissue water

Experimental Procedures

White male mice, weighing from 23 to 27 g were used They were divided into two main groups of which one was treated with oestradiol monobenzoate, 10 µg in 0 2 ml of arachis oil injected subcutaneously 6 and 4 days before the animals were killed Such treatment is known to cause an increase in the amount of ground substance in this particular mouse strain The two main groups were both divided into three smaller groups from which one was treated with an acidifying agent and the second with an alkalising agent, the third serving as control group Each of the six groups of treatment involved from 19 to 25 animals Acidosis was produced by one daily injection for three days of 1 ml of isotonic NH_4Cl i p Similar degrees of alkalosis were produced by one daily intraperitoneal injection on three consecutive days of 2 ml isotonic CH_3COO Na Clinically the animals were unaffected by the treatment Two hours after the last injection the animals were lightly anaesthetized with ether, and a midline laparatomy was performed, bloodsamples 600 to 800 µl, were drawn from the vena cava inferior for analyses of pH, bicarbonate, Na, K and Cl This procedure was terminated within three minutes after the start of anaesthesia The blood samples were kept in an ice bath and the pH values was determined within two hours of sampling Subsequently the animals were beheaded and bled The fur on the back was depilated and the skin excised for determinations of water, hexosamine, hydroxyproline and tissue electrolytes

On smaller separate groups of mice treated in the same way as described above, the pH of the whole blood and the plasma bicarbonate were determined in arterial blood taken from the carotid arteries after beheading unanesthetized animals to permit a comparison of these constituents in venous and arterial blood

Methods

The water contents of the tissues were determined after freeze drying the fresh samples to constant weight Likewise the fat-contents were detern[illegible] d treatment of the dry tissue with light petroleum ether and di-eth[illegible] it weight Tissue hexosamine contents were measured by the me[illegible] &

Table 4

Concentrations of electrolytes in plasma None of the values differ significantly from those of the control group

		Sodium meq l		Chloride meq/l		Potassium* meq/l	
	n	mean	s e m	mean	s e m	mean	s e m
Control	23	136	± 3 5	105	± 1 2	5 2	± 0 17
Acidotic animals	22	133	± 2 4	111	± 1 3	5 3	± 0 24
Alkalotic animals	25	138	± 3 4	102	± 1 9	4 8	± 0 17
Oestradiol treated	20	134	± 2 2	109	± 1 0	5 0	± 0 12
Oestradiol treated acidotic animals	19	138	± 3 0	107	± 2 3	5 0	± 0 16
Oestradiol treated alkalotic animals	22	139	± 2 4	101	± 1 5	4 9	± 0 18

* Some plasma samples showed slight haemolysis (approximately 20% of all samples) and were therefore not analyzed for potassium

200% increase in the amounts of sodium This agrees with the results of previous investigations (Hvidberg, Jensen-Holm & Langgård 1963) During acidosis the animals became dehydrated and the sodium excess was mobilized to an extent greater than can be explained by the loss of water Inducement of alkalosis did not alter the values significantly, but tended to exaggerate the findings in the oestradiol treated control group These findings are even more impressive when the rather variable quantitative response to the oestradiol treatment is taken into consideration In the non oestradiol treated groups the mean values after treatment with acidifying and alkalising agents matched well with the corresponding values for the oestradiol treated groups, but the changes were statistically insignificant In all instances the chloride content followed the water content

In table 6 are given the Na/Cl ratios for skin, plasma and the oestradiol-provoked 'oedema in all the groups of treatment The results indicate that treatment with oestradiol increased this ratio from 0 9 to 1 25 and further that the ratio in the oedema fluid of the oestradiol treated mice was 1 54, although the ratio in the plasma was only 1 23 During acidosis the ratios tend to normalize No changes in the ratio occurred in the plasma

Table 7 shows that changes in the electrolyte concentrations of the total tissue water and in the oestradiol provoked oedema were not caused by changes in plasma concentration, since this remain unchanged throughout the groups

The hydroxiproline values indicate that no changes in the collagen elements of the connective tissue had taken place

Table 2

Comparison between the pCO_2 values of arterial and venous blood

	pCO₂ in mm Hg										
	Arterial blood			Venous blood from anaesthetized animals							
	normal animals			normal animals				oestradiol treated animals			
	n	mean	range	n	mean		s e m	n	mean		s e m
Control	6	38	31–44	23	55	±	1.8	20	50	±	1.3
Acidotic animals	5	31	30–32	22	45	±	2.0	19	41	±	1.9
Alkalotic animals	5	39	33–45	25	55	±	1.6	22	54	±	1.8

venous blood are changeing parallelly, thus justifying the use of venous *blood for the purpose*

In table 2 are shown the corresponding values for the pCO_2 in mm of Hg

Table 3 summarizes the values for the pH of whole blood from normal and oestradiol-treated animals after treatment with acidifying or alkalising agents It appears that the changes induced by the treatments were intentionally slight, as was intended, but significant

In table 4 are given the concentrations of Na, K and Cl in the plasmas of all treated groups None of the values differ significantly from those of the control animals

In table 5 are shown the results summarizing the amounts of the various constituents in the skin Statistically significant changes are seen in the groups treated with oestradiol the oestradiol treated control group showed a 100% increase in the amounts of water and hexosamine but a

Table 3

The pH of whole blood the corrected bicarbonate of plasma and the concentration of haemoglobin in venous blood from normal and oestradiol treated mice after treatment with acidifying or alkalising agents (cfr table 1)

		pH			*Standard HCO_3* mEq/l			Haemoglobin g/100 ml		
	n	mean		s e m	mean		s e m	mean		s e m
Control	23	7.14	±	0.015	15.7	±	0.39	13.7	±	0.22
Acidotic animals	22	7.09	±	0.013	12.6+)	±	0.32	12.8	±	0.22
Alkalotic animals	25	7.20	±	0.014	18.6+)	±	0.46	13.1	±	0.33
Oestradiol treated	20	7.14	±	0.011	14.9	±	0.31	13.7	±	0.23
Oestradiol treated acidotic animals	19	7.10	±	0.016	12.0°)	±	0.45	13.0	±	0.26
Oestradiol treated alkalotic animals	22	7.19	±	0.014	17.6°)	±	0.44	13.7	±	0.29

+) significantly different from the control group ($P < 0.001$)

°) significantly different from the oestradiol treated control group ($P < 0$ [illegible]

Table 6

The ratio of sodium to chloride contents for skin plasma and oestradiol provoked oedema of the skin

Na/Cl ratio

	Skin		Plasma		Oestradiol provoked "oedema
	normal	oestradiol	normal	oestradiol	
Control	0 90	1 25	1 30	1 23	1 54
Acidotic animals	0 90	1 10	1 20	1 29	1 32
Alkalotic animals	0 90	1 32	1 35	1 38	1 64

Table 7

Concentrations of sodium and chloride in the total tissue water in oestrad ol provoked oedema compared with plasma concentrations

	Sodium meq/l			Chloride meq/l		
	In tissue water	In oedema fluid	(plasma)	In tissue water	In oedema fluid	(plasma)
Control	87 1	-	(136)	96 9	-	(105)
Acidotic animals	88 1	-	(133)	98 2	-	(111)
Alkalotic animals	89 1	-	(138)	92 5	-	(102)
Oestrad ol treated	132 5	179	(134)	106 3	116	(109)
Oestradiol treated and acidotic animals	116 2	151	(138)	105 4	114	(107)
Oestradiol treated and alkalotic animals	128 7	168	(139)	97 5	102	(101)

Discussion

Changes in the acid base status of the organism can presumably affect the electrolyte contents of the connective tissue It might even be expected that changes in the pH of blood and tissues are involved in the mechanism behind the deposition and mobilization of electrolytes in the connective tissue

Previous studies in this field are scarce, and the results are based on observations after inducement of highly unphysiological pH changes In our investigation the alteration in acid base status of the blood were moderate and presumably within the physiological range

Treatment with oestradiol of mice, of the particular strain used in this experiment, caused remarkable changes in the connective tissue Previous

Table 5

Amounts of water, hexosamine, hydroxiproline and electrolytes in the skin during acidosis, alkalosis and oestradiol treatment

Skin

	n	*Water* g/100 g*		*Hexosamine* mg/100 g*		*Hydroxiproline* g/100 g*		*Na* meq/100 g*		*Cl* mEq/100 g*		*K* mEq/100 g*	
		mean	s e m	mean	s e m	mean	s e m	mean	s e m	mean	s e m	mean	s e m
Control	23	289	± 4 1	403	± 11 1	6 88	± 0 112	25 2	± 0 55	28 0	± 0 44	18 7	± 0 52
Acidotic animals	22	279	± 2 9	413	± 8 4	7 00	± 0 116	24 6	± 0 48	27 4	± 0 37	17 5	± 0 48
Alkalotic animals	25	294	± 4 2	444	± 10 2	6 86	± 0 406	26 2	± 0 48	27 2	± 0 47	19 4	± 0 76
Oestradiol treated	20	572	± 24 8	1093	± 58 5	5 59	± 0 164	75 8	± 4 36	60 8	± 2 59	22 7	± 1 16
Oestradiol + acidosis	19	504+)	± 17 9	938	± 48 6	5 40	± 0 199	58 6°)	± 3 60	53 1×)	± 2 70	23 5	± 0 74
Oestradiol + alkalosis	22	592	± 22 2	1091	± 53 6	5 15	± 0 164	76 2	± 4 43	57 7	± 2 52	22 9	± 1 18

+) Significantly different from the oestradiol treated control group at P < 0 05
) – – – – – – – P < 0 001
) – – – – – – – P < 0 01

* Dry fat free tissue

Summary

By simultaneously determining the amounts of hexosamine, hydroxyproline, Na, K and Cl in skin and of Na, K and Cl in plasma, the effects of mild degrees of non respiratory acidosis and alkalosis have been studied in normal and oestradiol treated mice

Oestradiol treatment increased the amounts of water, hexosamine and electrolytes in the connective tissue and raised the ratio of sodium to water significantly During acidosis the greater part of this excess sodium was mobilized

The same tendencies could be observed in the groups not pretreated with oestradiol, but were not significant

The plasma values remained unchanged in all groups

The findings indicate that physiological changes in the acid base status of the tissues have an effect on the amounts of electrolytes in the connective tissue and that the amount of ground substance is closely related to this depot function

It is suggested that changes in the acid base status of the tissues are involved in the mechanism for controlling the electrolyte depots of connective tissue

Acknowledgements

This investigation was partly carried out in "The Laboratory for Investigation of the Pharmacology of the Connective Tissue" with a grant from the Danish National Association against Rheumatic Diseases

The authors thank E Prætorius, M D for valuable discussions For skilful technical assistance the authors thank Miss Lene Clausen and Miss Bente Wiegel

REFERENCES

Astrup P Om erkendelse af forstyrrelser i organismens syrebase stof [illegible]

Coll [illegible]

Eiler [illegible]

Hvic [illegible] pharmacol et toxicol 1963 20, 131 139

Levitt M F, L B Turner A Y Sweet & D Pandiri The response of bone connective tissue and muscle to acute acidosis *J Clin Investigation* 1956. 35 98 105

Martin C J & A E Axelrod A modified method for the determination of hydroxiproline. *Proc Soc Exp Biol & Med* 1953 83 461–462.

Prætorius, E. Organismens syre base balance bør vurderes i den Brønstedske terminologi *Ugeskr f Læger* 1954 116 751 758

investigations have shown that the amount of ground substance increases, as established by accumulation of water, acid mucopolysaccharides (SCHMIDT 1958) and electrolytes Sodium appears to be actively deposited (see table 7 and *cf* HVIDBERG, JENSEN-HOLM & LANGGÅRD 1963)

By examining the response to pH changes in these animals, information can be gained about the specifical role that the ground substance plays in electrolyte regulation

As shown in the tables, considerable mobilization of sodium results from acidifying treatment of oestradiol-treated mice The same changes as were seen after acidifying or alkalising treatment of the oestradiol treated mice appeared on a much smaller scale in the animals not pre treated with oestradiol, but the changes were not statistically significant

These results indicate that physiological changes in the acid base status of the tissues do indeed have an effect on the electrolyte status of con nective tissue The changes can, however, only be recorded when the amount of ground-substance in the connective tissue has been increased by artificial means This, on the other hand, points to the ground sub stance as the medium in which the deposition takes place

As far as changes in the concentration of plasma electrolytes are concerned, it must be remembered that during non-respiratory acidosis any fall in the base buffer must be compensated by a rise in the non buffer anions (e g Cl^+) or a fall in cations (especially Na^+) Likewise during non-respiratory alkalosis a rise in base buffer must be compensated by a fall in anions or by a rise in cations (*cf* SIGGAARD-ANDERSEN 1963) In our investigation the concentration of chloride in the plasma and in the oestradiol-provoked "oedema" decreased slightly during alkalising treatment, whereas no changes in the plasma electrolytes could be de monstrated on treatment with acidifying agents This in turn shows that the induced changes in acid-base status were only slight

LEVITT *et al* (1956) showed that profound acidosis in rats caused mobilization of the sodium excess from connective tissue TOBIN (1958) demonstrated that during acidosis and alkalosis in cats the distribution of sodium could be better correlated with the pH changes than with the sodium concentration in the extracellular space These observations are in good accordance with the results of the study presented here

On the basis of the above findings it seems reasonable to assume that changes in the acid-base status of the tissues are involved in the mecha nisms concerned with regulating the electrolyte depots in connective tissue

Seen in perspective the results are in good keeping with the well known fact that acidifying therapy has diuretic and saluretic side discussion of possible causal relations is beyond the scope o

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By simultaneously determining the amounts of hexosamine, hydroxyproline, Na, K and Cl in skin and of Na, K and Cl in plasma, the effects of mild degrees of non respiratory acidosis and alkalosis have been studied in normal and oestradiol treated mice

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REFERENCES

Astrup P Om erkendelse af forstyrrelser i organismens syrebase stofskifte *Ugeskr f Læger* 1954 116 [illegible]

Cotlov [illegible] an [illegible]

Eilers, [illegible] S [illegible]

Hvidb[illegible] Holm & H Langgård Sodium potassium and chloride in connective tissue *Acta pharmacol et toxicol* 1963 20 131 139

Levitt, M F, L B Turner A Y Sweet & D Pandiri The response of bone connective tissue and muscle to acute acidosis. *J Clin Investigation*. 1956. 35 98–105

Martin C J & A E Axelrod A modified method for the determination of hydroxiproline *Proc Soc Exp Biol & Med* 1953, 83 461–462

Prætorius, E. Organismens syre base balance bør vurderes i den Brønstedske terminologi *Ugeskr f Læger* 1954 116 751–758

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Seen in perspective the results are in good keeping with the well known fact that acidifying therapy has diuretic and saluretic side effects Any discussion of possible causal relations is beyond the scope of this paper

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From the Department of Pharmacology, University of Copenhagen
(Professor Knud O Møller, MD)

Sodium, Potassium and Chloride in Connective Tissue

By

Eigill Hvidberg, Jens Jensen Holm and Hans Langgård

(Received June 15 1963)

The loose interstitial connective tissue of the skin represents a large organ consisting of fibrils and a few cells embedded in ground substance The ground substance occupies a large part of the extracellular space Previous investigations dealing with the electrolyte contents of connective tissue have shown them to differ from those of the plasma and thus have indicated that the skin may serve as a reservoir of electrolytes Other authors, however, have found no evidence for such function of the connective tissue

It has previously been shown that the ground substance serves as a depot for water The purpose of the study described here was primarily to evaluate the possible existence of an electrolyte depositing function of the connective tissue and also to study the underlying mechanism

Methods

Material Skin from white male mice 6 to 7 weeks old weighing from 23 to 27 g

Groups of treatment

1) Control animals
2) 2 6% KCl added to the d[illegible]
3) [illegible]
4) [illegible]
5) [illegible] out on the 7th day the animals were given 7 ml of intraperitoneal isotonic saline in two doses at an interval of 3 hours Two hours after the last injection the animals were killed On an average 65% of the injected solution was retained at that time
6) Daily injections of intraperitoneal cortisone acetate 1 mg for 15 days
7) Ten µg of oestradiol monobenzoate in 0 2 ml of arachis oil injected subcutaneously 6 and 4 days before the animals were killed
8) A combination of treatments given to groups 3 and 7

Procedure The animals were beheaded and bled and the fur on the back was depilated 4 to 6 cm² of the skin were excised

Schmidt, A The influence of cortisone and oestradiol on the amounts of hexosamine and water in skin *Acta pharmacol et toxicol* 1958 14 350–358
Siggaard Andersen O The pH log pCO_2 blood acid base nomogram revised *Scand J Clin Lab Invest* 1962 14 598 604
Siggaard Andersen O The acid base status of the blood *Thesis* pag 66ff Munksgård Copenhagen 1963
Tobin R B Varying role of extracellular electrolytes in metabolic acidosis and alkalosis *Am J Physiol* 1958 195 685 692
Ussing H H P Kruhoffer J Hess Thaysen & N A Thorn The alkali metal ions in biology *Handbuch der experimentellen Pharmakologie* 13 Band Springer Verlag Berlin 1960 p 205
Winters R W, R T Whitlock J L DeWalt & L G Welt Effects of Alterations of the sodium concentrations of the serum upon the content of sodium in bone *Am J Physiol* 1958 195 697–701

Table I

Water hexosamine and electrolytes in the skin of mice under different treatments

	Water g/100 g dry defatted tissue			Na meq/100 g dry defatted tissue			Cl meq/100 g dry defatted tissue			K meq/100 g dry defatted tissue			Hexosamine mg/100 g dry defatted tissue			Na meq/l tissue water	Cl meq/l tissue water	Na/Cl ratio
	n	mean	s e m	n	mean	s e m	n*	mean	s e m	n	mean	s e m	n*	mean	s e m	mean	mean	
Control	43	299	± 4	43	27.5	± 0.3	21	31.3	± 1.0	43	21.4	± 0.6	21	536	± 10	92	104	0.88
2.6% KCl	10	288°)	± 6	11	27.7°)	± 0.6	4	27.5	± 0.3	11	19.7°)	± 0.7	4	510°)	± 14	97	95	1.00
2% NaCl	19	289°)	± 6	19	27.3°)	± 0.9	13	41.2+)	± 1.6	20	20.9°)	± 0.9	12	514°)	± 7	94	143	0.65
Dehydration 6 days	15	231+)	± 4	15	23.4+)	± 0.5	7	26.9+)	± 1.6	15	16.9+)	± 0.5	7	501°)	± 10	101	116	0.87
Dehydration and rapid rehydration	12	314°)	± 11	13	37.9+)	± 1.5	7	39.3+)	± 1.0	13	16.2+)	± 0.5	7	498°)	± 16	120	125	0.96
Cortisone 15 days	13	244+)	± 4	13	23.3+)	± 0.4	2	22.4 24.0		13	16.6+)	± 0.7	2	529 533		93	96	0.97
Oestradiol	11	530+)	± 16	11	59.5+)	± 2.2	5	53.6+)	± 0.5	11	24.3	± 0.5	5	1029+)	± 59	112	101	1.11
Oestradiol + 2% NaCl	10	569+)	± 30	10	66.1+)	± 4.2	4	59.8+)	± 2.9	10	23.4	± 1.1	5	1076+)	± 31	116	105	1.10

n = number of samples, each sample representing one animal
* = part of the samples representing a pool of tissue from 2 or more animals
+) = the difference from the value for the control group is statistically significant ($P < 0.001$)
°) = no statistically significant difference from the control group
s e m = standard error of the mean

Drying Freeze-drying to constant weight

Removal of fat Repeated extractions of the dry tissue with light petroleum and di-ethylether to constant weight

Determination of sodium and potassium 25 mg of the dry defatted tissue treated over night with 2 ml of concentrated HNO_3 and 0 5 ml of 30% H_2O_2 (wet combustion) After neutralization with ammonium hydroxyde and making up with water to 25 ml the contents of sodium and potassium were determined in a Beckman DU flame photometer and photomultiplier

Determination of chloride 30 mg of dried defatted tissue dissolved in 2 ml of 5 N KOH for 1 hour at 70 C After neutralization with 50% acetic acid, the chloride contents were determined by automatic potentiometric titration (Müller 1942) with silver nitrate (Radiometer's Titrator with a combined silver mercuro-sulphate electrode and saturated potassium sulphate)

Determination of Hexosamine 35 mg of dry defatted tissue hydrolysed for 15 hours in 4 ml of 2 N HCl at 100°C in sealed ampoules The Elson and Morgan method as modified by Blix (cf Schmidt 1958), was employed

In all analyses glass-distilled water was used

For some of the groups the concentrations of sodium, chloride and potassium were also determined in the plasma The blood was drawn from the *vena cava inferior* during ether anaesthesia The analyses were performed by the methods mentioned above, except for the hydrolyses

The results are given in meq, mg or g per 100 g of dry defatted tissue and in meq per litre tissue water The plasma values are expressed in meq per litre plasma

The importance of calculating on the basis of defatted tissue has been stressed before (Hvidberg 1959)

The mean values in the tables are given with their standard errors (s e m) the statistical analyses were based on the T – test

Results

The values for the control group (table 1) are in accordance with those given in the literature for other animal species, except for the amounts of potassium in the skin, which were found to be higher than usually recorded

Adding 2 6% KCl to the drinking water for 6 days did not alter any of the values

Adding 2% NaCl to the drinking water for 6 days produced a considerable increase in the chloride contents of the skin but no alteration in the sodium contents When 3% NaCl was given, the same changes occurred in the skin, but the plasma analyses showed about a 30% increase in the concentrations of both sodium and chloride (table 2)

Dehydration caused an equal decrease in sodium, chloride and water contents of the skin The potassium contents decreased by 20%

Acute rehydration with isotonic saline normalised the water contents, but raised the concentrations of sodium and chloride far beyond those of the control animals The potassium values corresponded to those of group no 4

Discussion

Little published information is available about the electrolytes in connective tissue The earliest extensive work in this field was carried out by MANERY, DANIELSON & HASTINGS (1938) A survey of early investigations has been given by ROTHMAN (1954) Only a few authors have examined the alterations in electrolyte contents induced by different treatments, and their results are often conflicting PADBERG (1910) demonstrated an excess of chloride in the skin of dogs after saline infusion The question of storing chloride in the skin has since been much debated ROWNTREE (1922) and SKELTON (1927) among others, arrived at similar results, whereas VOLK & FANTL (1939) and EGGLETON (1952), for example, disagreed with them DEBOER (1942) found that the chloride concentration increased by up to 60% in the skin of dehydrated dogs and that the concentration remained high for a long period after rehydration Thus the loss of water could not be correlated with an equivalent loss of chloride Feeding rats with large amounts of NaCl for about one year caused a decrease in water content and an increase in the chloride and sodium contents of aorta (HAIGHT & WELLER 1961) However, EGGLETON (1952) found no such an effect in the skin after infusion of saline, and COLE (1952) was only able to demonstrate in the skin of adrenalectomized rats a depot effect, which corresponded to the increase in extracellular space

It appears that the Na/Cl ratio is higher in the plasma (about 1 3) than in the skin (about 0 9, see table 2 and *cf* ROTHMAN 1954, SWEET, LEVITT & HODES 1961), which is predominantly a part of the extracellular space Further the chloride ion cannot be used for determining the extracellular space, in spite of the fact that it is almost exclusively extracellular These two facts may suggest an ability of the connective tissue to serve as a kind of reservoir for electrolytes

In our study 2% NaCl in the drinking water for 6 days lowered considerably the Na/Cl ratio in the connective tissue Provided that the Na/Cl ratio in the plasma is unchanged the increased chloride concentration in the connective tissue during salt loading could be explained by either deposition of chloride or a lack of sodium deposition, or by a combination of these To clarify this, the concentrations of sodium and chloride in plasma and skin were determined simultaneously on a small number of mice, which had received 3% NaCl in the drinking water for 6 days This higher concentration was chosen to accentuate the alterations in plasma concentrations From table 2 it is seen that, though the Na/Cl ratio in the plasma was a little elevated after this treatment, it was considerably decreased in the skin The treatment, however,

Table 2

Electrolyte values for plasma and skin of five mice treated with 3% NaCl in the drinking water for 6 days and of a control group analysed simultaneously, for which mean values are given with the standard errors

Mouse no	Hgb g/100 ml	Plasma Na meq/l	Plasma Cl meq/l	Skin water g/100 g dry tissue	Skin Na meq/100 g dry tissue	Skin Cl meq/100 g dry tissue	Na/Cl ratio plasma	Na/Cl ratio skin
1	17 3	218	138	238	24 4	42 2		
2	16 3	181	113	260	21 4	40 1		
3	14 2	214	164	234	26 0	36 5		
4	–	200	142	234	26 6	42 9		
5	–	184	128	242	19 5	33 5		
Average	15 9	199	137	242	23 6	39 0	1 45	0 60
Control (n = 23)	13 7±0 2	136±3 5	105±1 2	289±4 1	25 2±0 5	28 0±0 4	1 30	0 90

Animals treated with large doses of cortisone showed the same changes as the dehydrated group

Treatment with oestradiol caused a considerable increase in ground substance and in water content of the connective tissue This is in agreement with the results of previous investigations (SCHMIDT 1958, HVIDBERG 1959) The increase in chloride concentration was of the same magnitude as that in water content The sodium concentration, however, showed a further increase of about 15% The potassium values were only slightly elevated The concentrations of the electrolytes in the plasma remained unchanged during the treatment (table 3)

When 2% NaCl was added to the drinking water of the oestradiol treated mice, the concentrations of sodium, chloride, water and hexosamine were all higher than in the previous group

The hexosamine levels of all the groups agreed with those found in previous investigations (HVIDBERG 1959)

Table 3

Plasma concentrations of sodium chloride and potassium in two groups of mice analysed simultaneously one group was treated with oestradiol The control group was the same as for the figures in table 2

	n	Hgb g/100 ml mean s e m	Na meq/l mean s e m	Cl meq/l mean s e m	K meq/l mean s e m
Control	23	13 7 ± 0 2	136 ± 3 5	105 ± 1 2	5 2 ± 0 17
Oestradiol treated	20	13 7 ± 0 2	134 ± 2 2	109 ± 1 0	5 0 ± 0 12

a connection between the electrolyte depot and the ground substance of connective tissue In cartilage chondroitin sulphates (also acid muco polysaccharides) are known to deposit sodium (DUNSTONE 1959, FARBER 1960)

Thus it seems probable that the acid mucopolysaccharides of the ground substance are responsible for the same effect in the skin According to USSING *et al* (1960, pag 205) the macromolecules in the interstitial space act as mixed cation anion ion-exchangers This is possibly because the acidic and basic groups are placed at some distance apart

The depositing mechanism seems therefore to occur in the colloid structure of the connective tissue ground substance for water as well as for some of the electrolytes It should, however, be mentioned that other possible mechanisms exist and may act simultaneously Changes in the pH of the tissue buffer systems may explain part of the depot mechanism of the connective tissue In our investigation neither the pH nor the buffer capacity of the blood and the tissue were determined It is therefore impossible from the results reported here to tackle this problem Studies with this aim are in progress

Summary

By determinating the sodium, chloride, potassium, hexosamine and water contents of mouse skin, the problem of the connective tissue electrolytes has been approached Adding 2 6% KCl to the drinking water for 6 days did not change the values When 2 or 3% NaCl was given the chloride concentration increased by about 30% in the tissue, although the sodium concentration remained unchanged The Na/Cl ratio of the plasma was not affected by this treatment, since the concen trations of both ions were raised by about 30% Deprival of water for 6 days resulted in an equal decrease in sodium, chloride and water contents Acute rehydration of these animals with intraperitoneal isotonic saline raised both the sodium and the chloride concentrations by about 20% above those [illegible]
had the same effe [illegible]
which is known t [illegible] the connective tissue ground substance in this particular mouse strain doubled the contents of Na, Cl and water, but sodium retention was about 15% higher than that of chloride, in spite of unchanged plasma electrolytes

The results recorded here support the view that the connective tissue can deposit and mobilise sodium as [illegible]
stances The colloids of the ground su[illegible]
polysaccharides, presumably play an i[illegible] part in this process

produced an equal increase of approximately 30% in the sodium and chloride concentrations of the plasma At the same time the sodium concentration in the tissue water was unchanged whereas the concentration of chloride in the tissue water was increased by 50% The same change in the Na/Cl ratio of the tissue was found in the group that received 2% NaCl in the drinking water

The retention of sodium in the tissue after treatment with oestrogen hormones is greater than those of chloride and water This is not caused by a change *in the plasma* electrolytes, which remained unchanged (table 3) When oestradiol-treated, animals were given 2% NaCl in the drinking water as well, the sodium retention exceeded the values found in the group treated with oestradiol alone, whereas the changes found in the group given 2% NaCl but no oestradiol (*i e* dehydration and chloride retention) were absent The excess of sodium in the skin of the oestradiol-treated animals seems to be deposited in the increased amount of connective tissue

These findings can best be understood on the assumption of a shift of both sodium and chloride in the tissue independently of the plasma *concentrations Changes in the electrolyte dissolving parts of the extra* cellular water or changes in the structure of the colloids are possible explanations of the mechanism

It is noteworthy that KCl given in the drinking water instead of NaCl at the same molar concentration produced no changes in electrolyte concentrations of the tissue This focusses attention on the sodium ion

As stated, these observations agree with those of earlier investigations and with the points of view expressed in some recent publications (LEVITT *et al* 1956, SWEET, LEVITT & HODES 1961) Other authors however, hold different opinions GUNTHER, DULCE & SCHUTTE (1961) found the electrolyte changes in the skin after adrenalectomy to be primarily due to the changes in the water contents According to these authors the concentrations of sodium and chloride are always the same in a part of the extracellular space, named the sodium space and the chloride space The changes in the water contents should thus take place in a non-electrolyte dissolving part of the water, in close relation to the collagen fibrils However, the ' non solvent water" of the skin may be somewhat *constant* in amount (EILERS & LABOUT 1946) Moreover, MANERY, DANIELSON & HASTINGS (1938) and WIDDOWSON & DICKERSON *(1960)* found a constant ratio *of collagen to* water HVIDBERG *(1962) claimed* that the water depositing effect of the connective tissue was due to the colloids of the ground substance Hyaluronic acid plays an *important role*, since quantitative as well as qualitative changes in this substance affect water binding (HVIDBERG & JENSEN 1959)

In *our investigation* the results from the oes

Ussing, H H, P Kruhøffer J Hess Thaysen & N A Thorn *The alkali*

Widdowson E M & J W T Dickerson The effect of growth and function on the chemical composition of soft tissues *Biochem J* 1960 77, 30-43

Acknowledgements

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REFERENCES

Cole D F The effects of adrenalectomy on the electrolyte composition of the skin of the rat *Acta Endocrinol* 1952 11 9 20

DeBoer B *Alterations in the blood and in the water fat and chloride content of tissues during dehydration in the dog* Thesis University of Missouri 1942

Dunstone J R Some cation binding properties of cartilage *Biochem J* 1959 72 465–473

Eggleton M G The effect of injection of hypertonic salt solution on the chloride content of mammalian skin *J Physiol (Lond)* 1952 117 172–184

Eilers H & J W A Labout The non solvent water in hides *Proc Symp Fibr Protein* University of Leeds 1946 Pg 30–38

Farber S J Mucopolysaccharides and sodium metabolism *Circulation* 1960 21 941 947

Gunther Th H J Dulce & E Schutte Über den Einfluss der Nebenniere auf den Wasser Elektrolyt und Mucopolysaccharidgehalt der Haut *Arch f exper Path Pharm* 1961 242 201 206

Haight A S and J M Weller Plasma and tissue electrolytes of rats given excess sodium chloride in food *Proc Soc Exper Biol Med* 1961 108 209–211

Hvidberg E Effect of dehydration and fasting on the water binding mechanism of the connective tissue *Acta pharmacol et toxicol* 1959 16 28 37

Hvidberg E *Bindevævets vandindhold og grundsubstansens vandbinding* Thesis Odense Andelsbogtrykkeri 1962

Hvidberg E & C E Jensen Changes in molecular weight of acid mucopolysaccharides in connective tissue due to hormone treatment dehydration and age *Acta Chem Scand* 1959 13 2047 2056

Levitt M F L B Turner A Y Sweet & D Pandiri The response of bone connective tissue and muscle to acute acidosis *J Clin Invest* 1956 35 98 105

alyse 6 Ed Leipzig 1942

Hastings Connective tissue electro

375

Padberg ... der Haut als Chlordepot *Arch f exper Path Pharm* 1910 63 60–79

Rothman S *Physiology and biochemistry of the skin* University of Chicago Press 1954 Chapter 21

Rowntree L G The water balance of the body *Physiol Rev* 1922 2 116 169

Schmidt A The influence of cortisone and oestradiol on the amounts of hexosamine and water in skin *Acta pharmacol et toxicol* 1958 14 350–358

Skelton H The storage of water by various tissues of the body *Arch Int Med* 1927 40 140–152

Sweet, A Y M F Levitt & H L Hodes Kidney function body fluid compartments and water and electrolyte metabolism in the monkey *Amer J Physiol* 1961 201 975 979

nes, noradrenaline (NA) by the method of BERTLER, CARLSSON & ROSENGREN (1958), and dopamine (DA) by the method of CARLSSON & WALDECK (1958), modified by CARLSSON & LINDQVIST (1962)

Results and Discussion

The accumulation in the brain of O methylated catecholamine metabolites (MT and NM) brought about by nialamide was significantly enhanced by chlorpromazine and haloperidol (table 1) The threshold dose for this effect may be roughly estimated as 2 5 and 0 2 mg per kg for chlorpromazine and haloperidol, respectively In the animals treated with tranquillizers, the accumulation of MT was possibly enhanced by elevation of the ambient temperature to 30°, but in our limited material the difference was not statistically significant The levels of NA and DA were not significantly influenced by the tranquillizers In the experiments with haloperidol the DA level was about 25% higher when the mice were kept at room temperature than at 30° ($p < 0\ 005$), but no corresponding effect was observed in the experiments with chlorpromazine The material was not sufficient to permit any clear conclusions about the influence of temperature on the catecholamine levels

A few experiments were performed with promethazine and phenoxybenzamine (= bensylyt (NFN)), but in them no significant effects were observed

Our experiments show that low doses of chlorpromazine and haloperidol, i e, two major tranquillizers with different chemical structures but possibly similar modes of action, exerted a characteristic effect on the metabolism of brain catecholamines they enhanced the accumulation of the O methylated metabolites MT and NM brought about by MAO inhibition without influencing the levels of the catecholamines themselves Among several possible mechanisms the most likely appears to be that chlorpromazine and haloperidol block monoaminergic receptors [illegible]
brain a[illegible]
tryptam[illegible]
inhibito[illegible] precursors 5 hydroxytryptophan and dihydroxyphenylalanine It does not seem unreasonable to assume that this receptor blockade results in a compensatory activation of monoaminergic neurons This activation should lead to increased release of monoamines and consequently to increased formation of monoamine metabolites The reason why the increased monoamine release does not result in a decrease in monoamine levels may be that neuronal activation stimulates monoamine synthesis

Experiments are in progress in this laboratory to elucidate the mecha-

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From the Department of Pharmacology, University of Goteborg, Goteborg, Sweden

Effect of Chlorpromazine or Haloperidol on Formation of 3-Methoxytyramine and Normetanephrine in Mouse Brain

By

Arvid Carlsson and Margit Lindqvist

(Received June 13, 1963)

Unlike reserpine and similar agents, chlorpromazine and haloperidol have little or no effect on the monoamine levels of the brain. Large doses of chlorpromazine have been shown to interfere with the effects of reserpine and also with the effects of MAO inhibitors on brain monoamine levels (EHRINGER, HORNYKIEWICZ & LECHNER 1960, PLETSCHER & GEY 1962). It appears that these effects are at least partly due to hypothermia (CÓSTA, GESSA & BRODIE 1962, PLETSCHER 1963).

The effect described in this paper appears at a lower dose level and is not secondary to hypothermia.

Methods

White mice of either sex, weighing about 20 g were injected intraperitoneally with [illegible] dol [illegible]

nialamide alone. The animals were kept usually at room temperature [illegible] but in some experiments at 30°.

The brains of 9 mice were pooled and homogenized with 18 ml 0.4 N perchloric [illegible] of normetaneph- [illegible] thod of CARLSSON & LINDQVIST (1962). As an [illegible] ate was taken from the ion exchange column by means of 4 ml 0.1 N ammonia. This eluate [illegible] 5 N HCl. The MT was [illegible] pt that our chromatographic technique did not [illegible] oride [illegible]

The remaining part of the extract (about 5 ml) was taken

nism of the effect of chlorpromazine and haloperidol on the monoamines. It has been shown that chlorpromazine and haloperidol cause an increase in the levels of homovanillic and dihydroxyphenylacetic acids in rabbit brain (ANDEN, ROOS & WERDINIUS 1963).

It is evident that the possible usefulness of metabolite levels as indicators of neuronal activity merits further investigation.

Summary

The accumulation in mouse brain of the catecholamine metabolites 3-methoxytyramine and normetanephrine brought about by inhibition of monoamine oxidase was found to be enhanced by small doses of chlorpromazine and haloperidol, but phenoxybenzamine (bensylyt) and promethazine were ineffective. Hypothermia could be eliminated as a causative factor.

The effect is, it is suggested, due to a compensatory activation of monoaminergic neurons after blockade of monoaminergic receptors.

Acknowledgements

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For generous supplies of drugs we thank Leo Ltd., Hälsingborg (Chlorpromazine and Haloperidol), Recip Ltd., Stockholm (Promethazine), Smith Kline and French Labs., Philadelphia (Phenoxybenzamine), and the Swedish Pfizer Ltd., Näsbypark (Nialamide).

For technical assistance we thank Miss Anne Charlotte Lilljeqvist and Miss Erica Weber.

REFERENCES

Andén N. E., B. E. Roos & B. [illegible]
Bertler Å., A. Carlsson & E. [illegible] determination of adrenaline [illegible] *Scand.* 1958 44 273–297
Carlsson A. & M. Lindqvist. In vivo decarboxylation of α methyl DOPA and α methyl metatyrosine. *Acta physiol. Scand.* 1962 54 87–94
Carlsson A. & B. Waldeck. A fluorimetric me[illegible] dopamine (3 hyd[illegible]
C[illegible]
C[illegible]

Table 1.

Brain monoamine levels after various doses of chlorpromazine, haloperidol, phenoxybenzamine (bensylyt) or promethazine to mice pretreated with nialamide (100 mg per kg i p)

... killed 3 hours later

Figures in brackets indicate number of experiments
Each experiment was performed on 9 pooled brains

Drug mg/kg i p	Noradrenaline	Dopamine	Normetanephrine	3 Methoxytyramine
		Chlorpromazine		
25	–[3]	–[3]	0.16 (1)	0.36 (1)
5	0.49 ±0.050 (2)	1.21 ±0.145 (2)	0.13 ±0.035 (2)	0.53 ±0.045 (2)
5[1]	0.62 ±0.045 (2)	1.16 ±0.119 (3)	0.13 ±0.032 (3)	0.67 ±0.083 (3)
5[2]	0.55 ±0.045 (4)	1.18 ±0.080 (5)	0.13[4] ±0.021 (5)	0.61[4] ±0.060 (5)
2.5	0.54 ±0.095 (2)	1.16 ±0.075 (2)	0.07 ±0.010 (2)	0.41 ±0.010 (2)
1	0.61 (1)	1.34 (1)	0.08 ±0.010 (2)	0.29 ±0.014 (2)
		Haloperidol		
15	0.54 ±0.090 (2)	1.21 ±0.155 (2)	0.07 ±0.065 (2)	0.51 ±0.000 (2)
1	0.57 ±0.023 (4)	1.14 ±0.019 (4)	0.09 ±0.005 (4)	0.61 ±0.096 (4)
1[1]	0.57 ±0.027 (3)	0.91 ±0.031 (3)	0.08 ±0.009 (3)	0.78 ±0.056 (3)
1[2]	0.57 ±0.016 (7)	– –	0.08[5] ±0.005 (7)	0.69[4] ±0.065 (7)
0.2	0.63 ±0.030 (2)	1.34 ±0.035 (2)	0.07 ±0.015 (2)	0.43 ±0.005 (2)
0.05	0.53 ±0.055 (2)	0.87 ±0.040 (2)	0.08 ±0.010 (2)	0.31 ±0.045 (2)
		Phenoxybenzamine		
20	0.65 ±0.033 (3)	1.05 ±0.200 (3)	0.08 ±0.044 (3)	0.30 ±0.014 (3)
		Promethazine		
20	0.89 (1)	1.31 (1)	0.04 (1)	0.25 (1)
20 + 10	0.64 (1)	1.39 (1)	0.08 (1)	0.25 (1)
		Control		
0	0.63 ±0.044 (5)	1.24 ±0.150 (5)	0.06 ±0.011 (7)	0.29 ±0.035 (7)
0[1]	0.62 ±0.037 (4)	1.00 ±0.092 (4)	0.04 ±0.015 (4)	0.34 ±0.036 (4)
0[2]	0.62 ±0.028 (9)	1.13 ±0.097 (9)	0.05 ±0.009 (11)	0.31 ±0.025 (11)

1) The mice were kept at 30°
2) ... room temperature and 30 combined)
3) ... ol
4) ... n control
5)

Acta pharmacol et toxicol 1963, 20, 145–157

From Sct Hans Hospital Dept E, Roskilde, Denmark

Adrenergic Mechanisms and Amphetamine Induced Abnormal Behaviour

By

A Randrup, I Munkvad and P. Udsen

(Received July 11 1963)

It has often been assumed that the stimulating action of amphetamine is exerted through adrenergic mechanisms in the central nervous system (Dell 1960, Brodie, Spector & Shore 1959, Woolley 1962, Elkes 1958, van Rossum, van der Schoot & Hurkmans 1962, van der Schoot 1961)

Although criticized by Gelder & Vane (1962) in recent years, this assumption found some further support when it was shown (van der Schoot 1961, Maxwell 1959, Tripod 1952) that several adrenergic blocking agents antagonize amphetamine induced motor stimulation of mice (Tripod however, expresses doubt whether this antagonism is in fact caused by a specific anti adrenergic effect)

In our laboratory we have observed that rats given moderate doses of amphetamine (1 5–3 mg/kg) not only are hyperactive but the activity is also of a grossly abnormal stereotyped character This effect of amphetamine has not been studied earlier in detail, although abnormal behaviour that may be comparable (biting the cage wire, self [illegible]

[illegible]

[illegible] activities will be described, and it will be shown that the abnormality is not antagonized by several powerful adrenergic blocking agents

Such studies on the mechanism of the action of amphetamine on the central nervous system appear to be of interest for psychiatry (Karli 1960, Utena 1961), since in later years it has been reported repeatedly that in human subjects amphetamine and methamphetamine (in doses of roughly 1–10 mg/kg) can produce a psychotic state so closely resembling schizophrenia as often to cause mistakes (Connell 1958, Sano & Nagasaka 1956,

Ehringer, H, O Hornykiewicz & K Lechner Die Wirkung des Chlorpromazins auf den Katecholamin und 5 Hydroxytryptaminstoffwechsel im Gehirn der Ratte *Naunyn Schmiedeberg's Arch exp Path u Pharmakol* 1960, 239, 507–519
Pletscher, A, K F Gey & E Kunz Accumulation of exogenous monoamines in brain *in vivo* ...
"Binding Sites of Brain ...
March 1963 To be publi ...
Pletscher, A & K F Gey ...
monoamines and amino ...
prothixene In Paton, W D M and Lindgren, P, Editors Pharmacological analysis of central nervous action *Proceedings of the First International pharmacological Meeting* Pergamon Press, Oxford, 1962, 8 75 84

given ½ h before amphetamine (3 rats) and after amphetamine as with DHE (3 rats) 25 mg/kg were given ½ h before amphetamine (3 rats) and 100 mg/kg 1 h before (2 rats) Eight of these eleven rats died within 24 hours

Tranquillizers

Reserpine (Serpasil ®) Doses of 7 5 mg/kg i p + 15 mg/kg s c were given 13½ hours before the amphetamine injection (5 rats) All animals survived more than 24 hours but remained sedated and became extremely emaciated during subsequent days

Chlorpromazine (Prozil ®) Doses of 12 mg/kg s c were given after amphetamine as with DHE (7 rats) and simultaneously with amphetamine (2 rats) Smaller doses, 2 9 mg/kg, were also tried, both after and simultaneously with amphetamine (19 rats) All rats survived

Haloperidol Serenase ® (JANSSEN 1961) Doses of 0 06 mg/kg (2 rats), 0 12 mg/kg (5 rats) and 0 24 mg/kg (2 rats) were injected s c after amphetamine, as with DHE, 0 12 mg/kg was given simultaneously with amphetamine (2 rats) and 0 24 mg/kg one hour before amphetamine (2 rats) All animals survived.

Ethoxybutamoxane is described by SLATER & JONES (1958) as a drug that resembles known tranquillizing agents Doses of 3 mg/kg (in terms of the hydrochloride) were injected s c after amphetamine as with DHE (7 rats), 1 mg/kg was given 50 min before amphetamine (2 rats) and after amphetamine (2 rats) All animals survived

An amphetamine analogue

[illegible]

Amphetamine, chlorpromazine, haloperidol and reserpine were used in the standard solutions commercially prepared for injection into human subjects The other drugs were dissolved immediately before use Dibenzyline and dihydroergotamine were dissolved in propylene glycol 1N H_2SO_4 (95 5) hydergine in [illegible]

Neth[illegible]

[illegible]

and [illegible]

test [illegible]

instead of a [illegible]

was always [illegible]

animals ther [illegible] of the effect of amphetamine alone on the

day of each experiment T[illegible]

by the dealer on the same [illegible]

their weights by less than [illegible]

with any drug The numb[illegible]

the preliminary and in th[illegible]

Results

Description of the Abnormal Behaviour

In the first series of experiments 24 rats were given 3 mg/kg of amphetamine, and a characteristic abnormal behaviour was seen from about

BREITNER 1962, MASAKI 1956; MCCONNELL & MCILWAINE 1961, WALLIS, MCHARG & SCOTT 1949) The psychosis is usually the result of chronic abuse, but it has also been observed after a single dose (CONNELL 1958, p 33 and 42, WALLIS, MCHARG & SCOTT 1949)

Methods

The animals used were highly inbred white male rats 3–6 months old, weighing 160-350 g The rats were placed in separate cages, after injection of drugs, they were kept under constant observation by the same trained observer for four hours or longer

The drugs and their applications were

Amphetamine (Dexedrine ®) The doses were 1 5 and 3 mg/kg (in terms of d amphetamine base) injected subcutaneously Along with other drugs only the larger dose, 3 mg/kg, was used

α-Adrenergic blocking agents

Benzylyt (NFN), *phenoxybenzamine* (WHO), = *dibenzyline* ®, = *blocadren* ®) The largest dose used was 150 mg/kg i p + 300 mg/kg s c (in terms of the hydrochloride), and this dose was always applied before the amphetamine, the time intervals between the two drugs being 1 hr 10 min (4 rats), 1 hr 30 min (2 rats), 2½ hrs (4 rats), 3 hrs 20 min (2 rats) or 24 hrs (2 rats), three of these animals died within 24 hours Smaller doses varying from 37 5 to 225 mg/kg were also tried (14 rats) they were given i p or s c, from 21 hours before up to 40 min after amphetamine

Dihydroergotamine, DHE 45 Doses of 100 mg/kg (in terms of the methanesulphonate) were injected s c 30 min before amphetamine (4 rats) or 5 min before amphetamine (3 rats) Also 5 rats were given this dose after amphetamine at the time when the animals had just begun to lick the cage wire netting Two rats died within 24 hours, both had received DHE before amphetamine

Hydergine ® This substance is a mixture of equal parts of dihydroergocornine dihydro ergocristine and dihydro ergokryptine Doses of 100 mg/kg (in terms of the methanesulphonates) were injected s c 30 min before amphetamine (4 rats), 5 min before (3 rats) or after amphetamine as with DHE (3 rats) 50 mg/kg after amphetamine was given to two rats All animals survived

Phentolamine (Regitin ®) Doses of 300 mg/kg s c (referring to the methanesulphonate) were given 30 min before amphetamine (3 rats), 10 min before (3 rats) or after amphetamine as with DHE (3 rats) Six of these nine rats died within 24 hours Smaller doses were also tried, 200 mg/kg (2 rats) and 100 mg/kg (2 rats) were given after the amphetamine

β Adrenergic blocking agents

Pronethalol (BAN) (*Nethalide*, Alderlin ® 2 isopropylamino 1 (2 naphtyl) ethanol) (BLACK & STEPHENSON 1962, DORNHORST & ROBINSON 1962) Doses of 300 mg/kg (in terms of the hydrochloride) were injected s c 40 min before amphetamine (3 rats), 30 min. before (2 rats) and after amphetamine as with DHE (5 rats) One of the rats receiving pronethalol before amphetamine died within 24 hours 5 rats were injected after amphetamine with 150 mg/kg

Dichloroisopropylnoradrenaline, DCI (POWELL & SLATER 1958, BLACK & STEPHENSON 1962, MAYER 1962) Doses of 50 mg/kg s c (in terms of the hydrochloride) were

gradually replaced by more normal activities, such as *grooming, eating*

few short spells of grooming activity) at the wire netting of the cage, mostly the walls and ceiling, for periods of 43–99 min Sniffing the floor occurred, but only transiently The area covered was periodically restricted, but even then it extended over as much as a half to a whole cage wall (25–50 square inches) A few movements of the mouth were seen in two rats and constant licking in one, the remaining ten rats on this dose kept their mouths still and closed As the head was elevated, the mouth could be kept almost constantly under observation *E* A tendency to remain on one spot was noted, since it was not as persistent as with the 3 mg dose no time were recorded Walking was always in the forward direction None of the rats pressed or leaned the body against the wall at any time A direct comparison between the effect of the two doses was made by means of the experiment shown schematically in table I

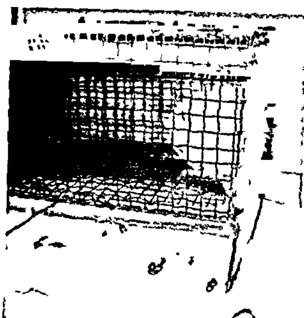
Effect of adrenol) tic substances

α Adrenergic Blocking Agents None of the four α adrenergic blocking agents, dibenzyline, dihydroergotamine, hydergine and phentolamine, inhibited the constant stereotyped behaviour induced by 3 mg/kg

Table 1

Rat No		6th June	13 June	21 June
1	Dose	3 mg/kg	saline	1 5 mg/kg[1]
	Behaviour	A B C[1]	normal	D E
2	Dose	3 mg/kg	saline	1 5 mg/kg
	Behaviour	A B C	normal	D E
3	Dose	1 5 mg/kg	3 mg/kg	saline
	Behaviour	A B	A B C	normal
4	Dose	1 5 mg kg	3 mg/kg	saline
	Behaviour	D E	A B C	normal
5	Dose	saline	1 5 mg/kg	3 mg/kg
	Behaviour	normal	D E	A B C
6	Dose	saline	1 5 mg/kg	3 mg/kg
	Behaviour	normal	D E	A B C

[1]) The behavioural characteristics are described in the text



Rat showing the abnormal behaviour described under items (A) (B) and (C) in the text

30 min to about 2 hours after the injection A representative rat is shown in fig 1, the behaviour is described below

A All the 24 rats sniffed constantly and actively, but only covered a small area, about two square inches, of the cage floor (20 rats) or the lower part of one of the walls (4 rats) Movements of the mouth and licking or biting of the cage wire netting were seen in all rats

B Total absence of locomotion Of the rats 22 took only a few steps (backwards only), and all the rats remained for a certain period (12–138 min, 18 rats more than 30 min) on the same spot, usually a corner of the cage *Only two animals walked (several times round the cage), always* backwards

C All the animals pressed their backs or their sides against a cage corner or a wall

The behaviour described above developed about 30 min after the injection and ceased about 2–3 hours later Before and after it was fully developed, a behavioural pattern similar to that observed after 1 5 mg/kg as described below, was seen in all rats It should be pointed out that during some part of these pre- and postphases, all the 24 rats showed some movement, walking forward and sniffing over the greater part of the wire netting of the whole cage The change from one behaviour pattern to the other occurred sometimes within a few minutes, on other occasions more gradually, but it could be readily recognized in all the rats Three to five hours after the injection the constant sniffing was

gradually replaced by more normal activities such as grooming, eating and drinking, the animals finally falling asleep

The pattern of behaviour elicited by 1 5 mg/kg (13 rats) is characterized as described below *D* All the rats sniffed constantly (only interrupted by a few short spells of grooming activity) at the wire netting of the cage, mostly the walls and ceiling, for periods of 43–99 min Sniffing the floor occurred but only transiently The area covered was periodically re stricted, but even then it extended over as much as a half to a whole cage wall (25–50 square inches) A few movements of the mouth were seen in two rats and constant licking in one, the remaining ten rats on this dose kept their mouths still and closed As the head was elevated, the mouth could be kept almost constantly under observation *E* A tendency to remain on one spot was noted, since it was not as persistent as with the 3 mg dose, no time were recorded Walking was always in the forward direction None of the rats pressed or leaned the body against the wall at any time A direct comparison between the effect of the two doses was made by means of the experiment shown schematically in table 1

Effect of adrenolytic substances

Table 1

Rat No	6th June	13 June	21 June
1 Dose Behaviour	3 mg/kg A B C [1]	saline normal	1 5 mg/kg[1] D E
2 Dose Behaviour	3 mg/kg A B C	saline normal	1 5 mg/kg D E
3 Dose Behaviour	1 5 mg/kg A B	3 mg/kg A B C	saline normal
4 Dose Behaviour	1 5 mg kg D E	3 mg/kg A B C	saline normal
5 Dose Behaviour	saline normal	1 5 mg/kg D E	3 mg/kg A B C
6 Dose Behaviour	saline normal	1 5 mg/kg D E	3 mg/kg A B C

[1] The behavioural characteristics are described in the text

amphetamine Two of the animals treated with hydergine and one treated with phentolamine were not observed to lick the cage wire, but exhibited only the constant *sniffing; all the other rats (numbers given under* "Methods") showed both sniffing and licking, similarly to the rats treated with amphetamine alone In view of the high (sublethal) doses and the time intervals between injections, which *gave the agents optimal* conditions for exerting their blocking action (see below), this result indicates strongly that inhibition of the stereotyped behaviour by blocking the α-adrenergic receptors is not possible This does not exclude the possibility of a depressing effect of the blocking agents on the general level of motor activity (see introduction to this paper), but this was not measured in our experiments (but, see below, experiment with dibenzyline and nethalide)

The eye slits of the rats treated with *amphetamine only* were always large and circular, and the eyeballs protruded These effects were absent from all animals treated also with dibenzyline or hydergine With amphetamine + dihydroergotamine or phentolamine the eye slits were extremely narrow These effects of the blocking agents lasted through the whole period of observation

Dibenzyline appeared to exert a taming effect on the rats, so that they scarcely resisted handling at the subsequent amphetamine injection The *taming effect* set in gradually, similarly to the adrenolytic effect of dibenzyline (PEART 1956), this was observed with eight of the animals receiving the maximum dibenzyline dose, 450 mg/kg At 30 min after the injection seven of the rats resisted handling, and were spitting and hissing, one hour after the injection only five of the animals behaved in this way, after *two hours they were all tame and easy to handle (but did not appear weak* or ill) The animals were still tame after the stereotyped behaviour had been induced by amphetamine In contrast, untreated rats and rats treated with amphetamine or with phentolamine and amphetamine did not become tamer on repeated handling

The rats injected with each of the blocking agents and saline were all quiet, grooming, drinking, sleeping and intermittent sniffing was observed Occasionally movement or twitching of the mouth was seen, but never licking or biting of cage wire Amphetamine with the solvent propylene glycol – H_2SO_4 (3 rats) caused the same behaviour and eye reaction as amphetamine alone

β-Adrenergic Blocking Agents

Like other sympathomimetic compounds without aromatic OH-groups, amphetamine has no β *adrenergic activity* (ARIENS & SIMONIS *1960)*

Table 2

Effects of adrenolytic substances and tranquillizers on rats injected with 3 mg/kg of d amphetamine

Substance	Maximal dose mg/kg	Inhibition of stereo typed behaviour induced by amphe tamine	Inhibition of the effect of amphetamine on the eyes	Other effects upon amphetamine treated rats
Dibenzyline	450 (14)[1]	0/14	12/14	Taming effect Prolongs stereo typed behaviour[2] and delays[3] onset
Dihydroergotamine	100 (12)	0/12	12/12	Delays onset of stereotyped be haviour[3]
Hydergine	100 (10)	0/10[7]	10/10	do
Phentolamine	300 (9)	0 9[7]	9/9	do
Nethalide	300 (10)	0/10[7]	0/10	Prolongs stereotyped behaviour[4]
DCI	50 (6)	0 6	0/6	
D benzyline + Nethalide	225 + 150[6] (5)	0/5	5/5	Decreases motor activity Pro longs stereotyped behaviour[5]
Reserpine	22 5 (5)	0/5	5/5	
Chlorpromazine	12 (9)	9/9	9/9	Increase followed by decrease of locomotion
Haloperidol	0 12 (7)	7/7	0/7	Increase followed by decrease of locomotion
Ethoxybutamoxane	3 (7)	7/7	7/7	do

[1]) The figures in parentheses show the numbers of animals given the maximum dose for further details see under Methods

[2]) Eight of the fourteen rats were still licking or biting more than [illegible] amphetamine one as late as the [illegible]

[3]) Delay 15–30 [illegible]

[4]) Three of the [illegible] hours after amph[illegible]

[illegible]

[illegible] g out in a few animals but not of sniffing see text

However, amphetamine releases nor adrenaline from brain and other tissues of the rat (McLEAN & McCARTNEY 1961, VANE 1960), and perhaps also adrenaline from the adrenals, it is conceivable that a β adrenergic action of the released amines is essential for producing the abnormal stereotyped behaviour of the rats

However, the two β adrenergic blocking agents nethalide and DCI did not antagonize this behavioural effect of amphetamine Only nethalide when given in maximal dose after aphetamine, appeared to reduce licking and biting in two of five animals, but these two animals did not interrupt their continuous sniffing All other animals were sniffing, licking or biting continuously, as on amphetamine alone The animals treated with nethalide and saline were quiet and much like those treated with α adrenergic blocking agents and saline, but DCI appeared to have some stimulating effect (this is also reported by v D SCHOOT 1961), and spells of discontinuous wire licking were observed with this drug It thus seems that blocking β adrenergic receptors does not inhibit the abnormal behaviour, and this conclusion is supported by our experiments with reserpine (see the next section)

The combined effect of α- and β adrenergic blocking was tested on five rats given both dibenzyline and nethalide, as shown in table 2 These animals licked and bit the wire netting of the cage floor continuously, but they appeared extremely quiet compared with animals receiving amphetamine alone For a period one animal was seen lying with virtually no movement of body or head, but gripping the wire with the teeth, while the tongue moved up and down The other animals were licking and biting with small, slow movements of head and jaws The animals were not weak or ill, and all survived Three rats treated with the two blocking agents and saline were quiet, but showed no licking, biting or sniffing This experiment thus demonstrates that a high level of motor activity is not a necessary prerequisite of the abnormal behaviour

Effect of tranquillizers

Our experiment with reserpine (see table 2) gave further evidence indicating that the release of adrenaline and nor adrenaline does not play an essential role in the production of abnormal behaviour by amphetamine The high dose of reserpine used (see "Methods") would ensure that nearly all adrenaline and nor adrenaline was removed from the tissues of the rat at the time when the amphetamine was injected (COSTA & PSCHEIDT 1961, HILLARP 1960, KARKI, PAASONEN & VANHAKARTANO 1959) In spite of the heavy sedation, all the animals reacted readily to amphetamine, became highly active, with the usual abnormal stereotyped activity

Six and a half hours after receiving the amphetamine, they were again heavily sedated During the period of abnormal activity their eye slits remained narrow, and the pupils of all the animals were extremely small, undoubtedly much smaller than those of untreated animals or animals treated with amphetamine alone The extreme narrowing of the pupils is a characteristic action of reserpine, which is most probably due to central [illegible]

tion has been made by ELDER & DILLE (1962) on cats given first reserpine and then LSD

In contrast to reserpine, three other tranquillizers that belong to other groups, chlorpromazine (a phenothiazine), haloperidol (a butyrophenone) and ethoxybutamoxane (a benzodioxane) were effective antagonists of amphetamine induced abnormal behaviour The effect was most clearly seen when the tranquillizers were given after amphetamine at the time when the rats had just begun licking the wire netting of a small area of the cage floor Within 2–15 minutes each of the three tranquillizers (chlorpromazine 12 mg/kg, haloperidol 0 12 mg/kg, ethoxybutamoxane 3 mg/kg) caused in all animals a change in behaviour, beginning with the animal's raising its head, sniffing over the walls and walking forwards (i e increase of locomotion, one rat, in which amphetamine induced backward walking reverted four minutes after chlorpromazine abruptly to forward walking) The licking decreased or ceased, and shortly after the sniffing diminished (no longer continuous), and the animals became quieter

Behavioural effect of the tryptamine analogue of amphetamine

The fact that various amines, including some tryptamines, show motor stimulating activity (v D SCHOOT 1961, VANE *et al* 1961, ALLES & FEIGEN 1941) indicates that of amphetamine not to be sufficiently specific to be classed as adrenergic We wondered if other amines also would produce the abnormal behaviour of rats and tested α methyltryptamine, the tryptamine analogue of amphetamine It was found that with 40 mg/kg (of the D, L form) a behaviour pattern could be elicited that closely resembled the amphetamine induced pattern ABC (see above) with all its characteristic features There was also a pre phase with constant sniffing but this sniffing was almost exclusively at the floor, whereas with amphetamine it was at the walls and the ceiling The duration of the abnormal behaviour was much longer with α methyltryptamine and a post phase was not seen It also lasted longer before the animals became active (1–2 hours), and during this period they were quiet but awake

Discussion

For the evaluation of these behavioural studies it is important to know whether or not the blocking agents are able to cross the blood brain barrier and to exert an action on the central nervous system The relevant information can be found in standard pharmacological textbooks and in some recent original papers Dibenzyline has been demonstrated in brain tissue after intravenous injection (BRODIE ARONOW & AXELROD 1954, table 6) and there is strong evidence indicating several central actions (NICKERSON 1959, MUNOZ & GOLDSTEIN 1961, ELDER & SCHELLENBERGER 1961, SINGER 1961), also the taming effect observed by us (and by ELDER & DILLE 1962 in cats) suggest a central effect of this drug

It has also been demonstrated that dihydroergotamine and hydergine (NICKERSON 1959, SANDOZ) as well as phentolamine (TRIPOD 1957, SICUTERI, MICHELACCI & FRANCHI 1962) exert central effects The β-adrenergic blocking agents nethalide and DCI have both been demonstrated in brain tissue after systemic application, and there is some evidence for central actions of these two drugs (see references given under "Methods")

The time intervals between the blocking agents and amphetamine were chosen with the intention of creating optimal conditions for developing the adrenergic blocking action Injection of the blocking agents after amphetamine was done primarily because tranquillizer activity was most easily discovered by this mode of injection

With this background, our experiments with adrenergic blocking agents and with reserpine suggest that if the α- or β adrenergic receptors known to exist in the periphery exist also in the central nervous system, they would not be involved in the development of amphetamine induced abnormal behaviour of rats

Admittedly, the tranquillizers chlorpromazine and ethoxybutamoxane which do antagonize abnormal behaviour also exert an α adrenergic blocking action If the latter property, however, is to be held responsible for their anti amphetamine action, it becomes hard to understand why the four classical strongly α adrenergic blocking agents that exert other actions upon the central nervous system do not share the anti amphetamine activity Further, the strongest amphetamine antagonist used in our experiments was haloperidol which is a tranquillizer with extremely low α adrenergic blocking activity (JANSSEN 1961) It therefore seems most likely that the anti amphetamine activity of both chlorpromazine ethoxybutamoxane and haloperidol may be due to the tranquillizing activity they have in common

Tranquillization and α adrenergic blocking thus seem to be due to two different properties of the compounds, a conclusion in agreement with

views expressed by NICKERSON (1959), but contrary to those of some other authors (BRODIE, SPECTOR & SHORE 1959, SLATER & JONES 1958)

Our conclusions about the adrenergic receptors receives further support from the observation that the behavioural effects of amphetamine are so closely mimicked by the tryptamine analogue of amphetamine, α methyl tryptamine This result might be considered as support for the hypothesis of Vane (GELDER & VANE 1962, VANE 1960), who has suggested that amphetamine may act upon tryptamine receptors in the central nervous system Here it must however, be recalled, that the adrenergic blocking agents dibenzyline, dihydroergotamine and hydergine also possess antitryptamine (antiserotonin) activity (VANE 1960, GADDUM & PICARELLI 1957, GADDUM *et al* 1955, DOEPFNER & CERLETTI 1958, FANCHAMPS *et al* 1960), and we have observed that the strong tryptamine antagonist Deseril or UML-491, Sandoz (DOEPFNER & CERLETTI 1958, FRANCHAMPS *et al* 1960) even at the extremely high subcutaneous dose of 100 mg/kg, does not abolish the stereotyped behaviour induced by either amphetamine or α methyltryptamine

A detailed account of our work on tryptamine mechanisms is being prepared for publication later, but the experiments reported in this paper have led us to think that the receptors involved in the production of the abnormal stereotyped behaviour after amphetamine and α methyltryptamine are different from both the adrenergic and tryptamine (serotonin) receptors recognised in peripheral systems These new receptors may occur only in the central nervous system and appear to have a specificity that permits the action of amines from both the adrenaline and the tryptamine series

Summary

Two patterns of abnormal stereotyped behaviour induced in rats by moderate doses of amphetamine are described The abnormal behaviour is inhibited by several tranquillizers, but not by ordinary α or β-adrenergic blocking agents It can also be produced by the tryptamine analogue of amphetamine, α methyl tryptamine, but is not inhibited by some tryptamine (serotonin) antagonists In the light of these findings the specificity of the receptors involved in the behavioural effects of amphetamine and α methyltryptamine is discussed

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REFERENCES

Alles, G. & G Feigen Comparative Physiological Actions of Phenyl-, Thienyl- and Furylisopropylamines *J Pharmacol Exper Therap* 1941, 72, 265–275

Ariens, E J & A M Simonis: Autonomic Drugs and Their Receptors *Arch int pharmacodyn* 1960, 127, 479–496

Black, J & J Stephenson Pharmacology of a New Adrenergic Beta-Receptor-Blocking Compound (Nethalide) *Lancet* 1962, II, 311–314

Breitner, C Discussion, in *Annals N Y. Acad Sci* 1962, 96, 90

Brodie, B, L Aronow & J Axelrod The Fate of Dibenzyline in the Body and the Role of Fat in its Duration of Action *J Pharmacol Exper Therap* 1954, 111, 21–27

Brodie, B, S Spector & P Shore Interaction of Drugs With Norepinephrine in the Brain *Pharmacol Revs* 1959, 11, 548–563

Connell, P H Amphetamine Psychosis *Maudsley Monographs*, No 5 London 1958

Costa, E & G Pscheidt Correlations Between Active Eyelid Closure and Depletion of Brain Biogenic Amines by Reserpine *Proc Soc Exper Biol Med* 1961, 106, 693–696

Dell, P : In *Adrenergic Mechanisms*, p 398, edited by Vane, J, G Wolstenholme & M O'Connor, Ciba Symposium, Churchill London 1960

Doepfner, W & A Cerletti Comparison of Lysergic Acid Derivatives and Antihistamines as inhibitors of the Edema Provoked in the Rat's Paw by Serotonin *Int Arch Allergy* 1958, 12, 89–97

Dornhorst, A & B Robinson Clinical Pharmacology of a Beta-Adrenergic-Blocking Agent (Nethalide) *Lancet* 1962, II, 314–317

Elder, J & J Dille An Experimental Study of the Participation of the ... Diethylamide (LSD) ... 62, 136, 162–168

Elde ... ic Acid Diethylamide (LSD) Induced Hyperthermia Pharmacologist 1961, 3, 59

Elkes, J In *Neurological Bases of Behaviour*, p 326 and 330, edited by Wolstenholme, G, C O Connor Ciba Symposium, Churchill London 1958

Fanchamps, A, W Doepfner, H Weidmann & A Cerletti Pharmakologische Charakterisierung von Deseril ®, einen Serotonin-Antagonisten *Schweiz Med Worschr* 1960, 90, 1040–1046

Gaddum, J, K Hameed, D Hathway & F Stephens Quantitative Studies of Antagonists for 5-Hydroxytryptamine *Quart J Exper Physiol* 1955, 40, 49

Gaddum, J & Z Picarelli Two Kinds of Tryptamine Receptor *Brit J Pharmacol* 1957, 12, 323–328

Gelder, M & J Vane Interaction of the effects of Tryptamine Amphetamine and Reserpine in Man *Psychopharmacologia* 1962, 3, 231 241

Hillarp, N Effect of Reserpine on the Nucleotide and Catecholamine Content of the Denervated Adrenal Medulla of the Rat *Nature*, 1960 187, 1032

... Temperature on Amphe... 1960, 1, 210–220

... ifferences I Correlation ... isitivity to Stimulant and ... 1958, 123, 206–211

Janssen, P Vergleichende pharmakologische Daten über sechs neue basische 4 – Fluorobutyrophenon – Derivate *Arzneimittel-Forsch* 1961, 11, (819-824 & 932 938) See section D

Maxwell, D The Activity of some Adrenaline Antagonists in Test for

May

Mc

Mc

Munoz C & L. Goldstein Influence of Adrenergic Blocking Drugs Upon the Eeg Analeptic Effect of dl Amphetamine in conscious Unrestrained Rabbits *J Pharmacol Exper Therap* 1961, 132, 354–359

Nickerson, M Blockade of the Action of Adrenaline and Noradrenaline *Pharmacol Revs* 1959, 11, 443-461

Peart, W. The β-Haloalkylamines In *Hypotensive Drugs* p 69-80 edited

s by a
1958,

Sandoz Mutterkornalkaloide

Singer, J The Effects of Ephinephrine, Chlorpromazine and Dibenzyline Upon the Fright Responses of Rats Under Stress and Non Stress Conditions Ann Arbor Mich Univ Microfilms Inc

Van Schoot, J *Wekaminen* Thesis 1961, University of Nijmegen, Holland

Vane, J R In *Adrenergic* ... & M O C

Vane, J R, Receptor

Van Rossum of Cocai 229 231

Woolley, D *The Biochemical Bases of Psychoses* Wiley, N Y 1962. (see p 201)

(Regitine); Dr. J. Black, Imperial Chemical Industries (Nethalide); Dr. I. H. Slater, Eli Lilly & Co. (ethoxybutamoxane); Dr. H. Collier, Parke Davis & Co. (α-methyltryptamine); Dr. V. Larsen, Dumex (information).

Mrs. Grethe Jensen's excellent care of the animals has contributed much to our work.

REFERENCES

Alles, G & G Feigen· Comparative Physiological Actions of Phenyl-, Thienyl- und Furylisopropylamines *J Pharmacol Exper Therap* 1941, 72, 265–275

Ariens, F J & A M Simonis Autonomic Drugs and Their Receptors

/ Adrenergic Beta-Recep-
962, 11, 311–314

Breitner, C. Discussion, in *Annals N Y Acad Sci* 1962, 96, 90

Brodie, B, L Aronow & J Axelrod The Fate of Dibenzyline in the Body and the Role of Fat in its Duration of Action *J Pharmacol Exper Therap* 1954, 111, 21–27

Brodie, B, S Spector & P Shore Interaction of Drugs With Norepinephrine in the Brain *Pharmacol Revs* 1959, 11, 548–563

Connell, P H Amphetamine Psychosis *Maudsley Monographs*, No 5 London 1958

Costa, F & G Pscheidt Correlations Between Active Eyelid Closure and Depletion of Brain Biogenic Amines by Reserpine *Proc Soc Exper Biol Med* 1961, 106, 693–696

Dell, P In *Adrenergic Mechanisms*, p 398, edited by Vane, J, G Wolstenholme & M O Connor, Ciba Symposium, Churchill London 1960

Doepfner, W & A Cerletti Comparison of Lysergic Acid Derivatives and ... of the ... Provoked in the Rat's Paw

rgic-

f the

Elde

rmacologist 1961, 3, ...

Elke ... *iour*, p 326 and 330, edited by Wolstenholme, G, C O Connor, Ciba Symposium, Churchill London 1958

Fanchamps, A, W Doepfner, H Weidmann & A Cerletti Pharmakologische Charakterisierung von Deseril (R), einem Serotonin-Antagonisten *Schweiz Med Wochschr* 1960, 90, 1040 1046

Gaddum, J, K Hameed, D Hathway & F Stephens Quantitative Studies of Antagonists for 5-Hydroxytryptamine *Quart J Exper Physiol* 1955, 40, 49

Gaddum, J & Z Picarelli Two Kinds of Tryptamine Receptor *Brit J Pharmacol* 1957, 12, 323–328

Gelder, M & J Vane Interaction of the effects of Tryptamine Amphetamine and Reserpine in Man *Psychopharmacologia* 1962, 3, 231 241

Hillarp, N Effect of Reserpine on the Nucleotide and Catecholamine Content of the Denervated Adrenal Medulla of the Rat *Nature*, 1960, 187, 1032

– Temperature on Amphe
1960, 1, 210 220
ifferences I Correlation Between Control Locomotor Activity and Sensitivity to Stimulant and Depressant Drugs *J Pharmacol Exper Therap* 1958, 123, 206 211

When the lower dose was given, morphine was injected as 3 mg/ml in 0 9% sodium chloride solution

Compound 48/80 and polymyxin B sulphate were administered intraperitoneally as 2.35 mg/ml of compound 48/80 and 1 25 mg/ml of polymyxin B in 0 9% sodium chloride solution. The treatment schedules for compound 48/80 and polymyxin B were the same as those previously described by PARRAT & WEST (1957) with slight modifications *Compound 48/80* was given in a total amount of 22 mg/kg in 7 doses over 4 days The total amount of *polymyxin B* administered was 35 mg/kg, given in 6 doses over 4 days Some of the rats died during the 4 days of treatment

Experiments To determine histamine we used total brains of single animals and about 1 g of skin (outer layer + inner layer), which was excised after depilation from the forepart of the back of the animals The tissues were treated and their contents of histamine determined as described by NORN (1963) In contrast to the results of experiments with dogs (ADAM 1961), we found no measurable amounts of histamine in the rat hypophysis, it was therefore left *in situ* when the brain was taken out

To evaluate the susceptibility of our animals to the action of strong histamine liberators, one group was treated with compound 48/80 and one with polymyxin B The animals were allowed to recover for 48 hours after the last dose, they were then killed and the amounts of histamine in brain and skin were determined

The effect of repeated morphine administration on the histamine contents of brain and skin was investigated. For this purpose we used morphine tolerant rats, which were killed 48 hours after the last daily dose of morphine (150 mg/kg) To evaluate any possible effect of a single toxic dose of morphine on the histamine contents of brain and skin, 150 mg/kg of morphine were given to normal rats, which were killed 48 hours later

The effect of 'analgaesic" doses of morphine on the histamine contents of brain and skin was also studied According to JÓHANNESSON & SCHOU (1963b), most of the normal rats given 8 mg/kg of morphine showed an increased pain threshold after 30 minutes We therefore injected normal rats with this dose of morphine and killed them 30 minutes later For comparison, some of the rats given 150 mg/kg of morphine were also killed 30 minutes after the injections Histamine was determined in brain and skin as usual

The histamine releasing capacities of compound 48/80, morphine chloride and polymyxin B sulphate were studied *in vitro* in a suspension of mast cells derived from the rat peritoneum For this purpose 14 rats were injected with 18 ml of a buffered solution consisting of 1 6 × 10^{-1}M NaCl, 3 × 10^{-3}M K Cl, 9 × 10^{-4} M anhydrous $CaCl_2$ plus 10% (v/v) of the Sørensen phosphate buffer (4 8 × 10^{-2}M Na_2HPO_4, $2H_2O$ + 3 × 10^{-2}M KH_2PO_4) The rats were killed about 30 seconds after the injection The abdominal wall was opened, and approximately 12 ml of the injected solution, now containing mast cells, were removed from the peritoneal cavity The withdrawn specimens were pooled, and the histamine concentrations were determined The concentration of histamine in the pooled mast cell suspension was 1 15 µg/ml Samples of 10 ml each were taken out of the pool and incubated with [illegible]
48 80, morphine or [illegible]
fuged and the hista [illegible]
supernatant The a [illegible]
as a percentage of t [illegible] ml sample

Determination of histamine was performed by the method of LOWRY *et al* (1954), modified by NORN (1963), giving an average recovery of about 90% from the brain. All results are expressed as µg histamine base per g tissue

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From the Department of Pharmacology, University of Copenhagen
(Professor Knud O Moller, M D)

The Effect of Morphine on the Histamine Contents of Brain and Skin in the Rat

By

Torkell Jóhannesson and Svend Norn

(Received June 19 1963)

In the literature morphine is classified as a histamine liberator in peripheral connective tissues (NASMYTH & STEWARD 1950, FELDBERG & PATON 1951, MONGAR 1956, MILTHERS & SCHOU 1958) Results of experiments demonstrating the histamine liberating capacity of morphine in the brain, however, do not appear to be so far available In the investigation reported below, we have therefore studied the effect of morphine on the histamine content of brain and have compared it with the liberation of histamine in the skin of rats The influence of *compound 48/80* and polymyxin B on the histamine contents of both brain and skin has been studied Further, the histamine liberating capacities of compound 48/80, polymyxin B and morphine have been compared *in vitro* in a suspension of cells derived from the rat peritoneum

Experiments and methods

Adult male albino rats (130–220 g) were used Morphine tolerance in the rats was produced as previously described (JÓHANNESSON 1962) The last daily dose amounted to 150 mg/kg morphine The daily injections of morphine were given subcutaneously and medianly 2–3 cm above the root of the tail

Doses and injection solutions For the experiments proper morphine was given intraperitoneally in amounts of 150 mg/kg or 8 mg/kg When the higher dose was given, morphine was administered as the chloride as an aqueous solution of 40 mg/kg

Table 2

[illegible] given and the p values for the statistical comperation between the control and the treated groups The statistics are carried out according to the t test

Control group		Morphine tolerant rats		Rats given single injections of 150 mg/kg morphine	
Brain	Skin	Brain	Skin	Brain	Skin
6 4	23 4	8 2	36 6	9 1	41 3
7 3	23 5	9 7	34 6	6 6	38 0
7 4	30 2	10 3	35 6	7 8	40 6
8 7	21 3	9 9	42 9	9 2	34 4
7 5	32 4	11 7	36 9	9 0	28 5
9 1	20 8	8 6	35 8	8 2	22.6
8 4	22 5	8 1	31 1	8 4	29 9
8 2	28 2	6 9	36 8	9 4	18 7
8 8	30 2	6 9	43 5	–	–
8 2	28 3	8 1	38 8	–	–
9 0	26 4	9 0	35 2	–	–
8 3	23 4	9 4	24 4	–	–
10 0	36 1	10 4	26 8	–	–
8 6	41 4	7 5	21 5	–	–
8 9	25 8	6.9	18 7	–	–
8 3	29 4	7 9	25 4	–	–
8 3	22.6	8 4	24 0	–	–
8 0	25 4	7 4	26 2	–	–
8 7	24 2	8 0	25 8	–	–
8 7	26 1	9 1	28 6	–	–
8 9	26 1	8 8	23 6	–	–
7 8	–	–	–	–	–
8 3 ± 0 2	27 0 1 1	8 6 ± 0 3	31 1 ± 1 6	8 5 ± 0 3	31 8 ± 2 9
		0 4 > P > 0 3	P = 0 05	0 8 > P > 0 7	0 2 > P > 0 1

group a slightly significant (P = 0 05) *increase* in histamine was found in the skin In the skin, the individual results were variable, with a large standard deviation

Six normal rats received 150 mg/kg and another six 8 mg/kg of morphine They were killed 30 minutes later, and histamine was determined in brain and skin Histamine was found in the same amounts as in brain and skin of 4 control animals

The histamine releasing capacities of compound 48/80, morphine and polymyxin B in a suspension of mast cells derived from the rat peritoneum

Compound 48/80 was applied to the suspension at five different concentrations morphine and polymyxin B each in three The results are shown in fig 1

Results

The effect of compound 48/80 and polymyxin B on the histamine contents of brain and skin

Seven rats received compound 48/80, and 5 rats received polymyxin B by intraperitoneal injections in increasing doses for 4 days The rats were killed 48 hours after the last dose and histamine was determined in brain and skin For comparison, histamine was determined in brain and skin of 4 rats killed without any previous administration of drugs The results are given in table 1 On an average, compound 48/80 and polymyxin B reduced the histamine contents of the skin by 75% and 70% respectively These substances had, however, no effect on the histamine content of the brain

The effect of morphine on histamine contents of brain and skin

Twenty one morphine tolerant rats were killed 48 hours after the last dose Eight normal rats received 150 mg/kg of morphine by single intra peritoneal injections They were killed 48 hours later For comparison 22 rats were killed without any previous administration of drugs Histamine was determined in brain and skin of the rats The results are given in table 2

The results show that morphine, whether given repeatedly or as a single toxic dose did not significantly affect the histamine contents of rat brain or skin ($P > 0\ 02$, by the t test) However in the morphine tolerant

Table 1

Concentrations of histamine in brain and skin of rats given repeated intraperitoneal injections of compound 48/80 and polymyxin B and killed 48 hours after the last dose The histamine contents are expressed as µg histamine base per g tissue The means ± s e m are given and the P values for differences between control and treated groups as calculated by the *t* test

Control group		Rats treated with compound 48/80		Rats treated with polymyxin B	
Brain	Skin	Brain	Skin	Brain	Skin
12 0	40 0	12 1	12 7	11 7	12 1
12 4	41 0	12 0	8 2	12 5	12 1
11 3	45 0	11 8	11 7	11 1	11 7
13 4	41 3	10 9		13 6	13 3
	–	11 7	9 0	12 0	11 5
–	–	11 7	12 0		
–	–	11 8	8 2		
12 3 ± 0 4	41 8 ± 1 1	11 7 0 2	10 3 ± 0 9	12 2 ± 0 4	12 1 ± 0 3
–	–	0 3 > P > 0 2	P < 0 001	0 9 > P > 0 8	P < 0 001

It is therefore highly probable that the failure of compound 48/80 and polymyxin B to release histamine from the brain is due to a failure in permeation of these substances into the brain from the blood

PARRATT & WEST (1957) administered 140 mg/kg of morphine intra peritoneally in 7 doses over 4 days Eight hours after the last dose they found a reduction in histamine to 60–80% of the control value in most tissues Details of the experiments were however not given

Our results for the effect of morphine on the histamine contents of tissues are not in agreement with those of PARRAT & WEST We found no reduction in histamine contents of brain or skin in normal rats whether given a single toxic dose (150 mg/kg) or a single analgaesic dose (8 mg/kg) of morphine intraperitoneally Further histamine was found in the same amounts in the brains of morphine tolerant rats and of normal rats The same was also true of the skin (table 2)

PARRATT & WEST (1957) found that the return of histamine to the tissues

[illegible] of morphine For this reason it therefore seems pro bable that no histamine was released at all by morphine from the brain or the skin of our animals

The results of our experiments *in vitro* confirm that morphine may indeed liberate histamine but only when applied at extremely high

[illegible] μg/g morphine in the brain 30 minutes after intraperitoneal administration of 10 mg/kg Further the concentrations of morphine seldom exceeded 40 μg/g brain even when highly toxic amounts of the drug were administered to rats (JOHANNESSON 1962) From our experi ments it is therefore concluded that neither the development of morphine tolerance nor the analgaesic action of morphine in rats is connected with liberation of histamine

Summary

1 The effect of compound 48/80 polymyxin B and morphine on the histamine contents of brain and skin of rats was investigated For com parison the histamine releasing capacities of these substances were tested *in vitro* in a mast-cell suspension

2 Injections of compound 48/80 and polymyxin B reduced the hista mine contents of skin by 70–80% the histamine contents of brain re maining unchanged

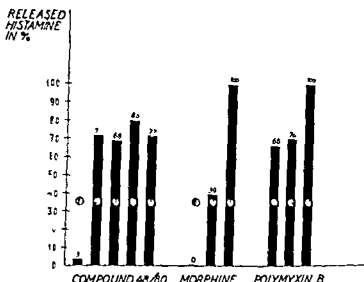


Fig 1 The amounts of histamine released, as percentages of total contents in a suspension of mast cells derived from the rat peritoneum, by compound 48/80 polymyxin B or morphine
Columns 1–5 indicate the percentages of histamine released by compound 48/80 applied at 5 concentrations to the suspension The concentrations were 0 05–0 5–5 0–50–500 µg per ml
Columns 6–8 indicate the percentages of histamine released by morphine at the concentrations 10–100–1000 µg per ml
Columns 9–11 indicate the percentages of histamine released by polymyxin B at concentrations 0 5–5 0 50 µg per ml

When applied at a concentration of 0 5 µg/ml to the suspension, compound 48/80 and polymyxin B released 72% and 66% of the total histamine in the suspension, respectively Applied at a concentration of 100 µg/ml to the suspension, morphine released 39% of the total amount of histamine

Discussion

Histamine was found in much lower amounts in brain than in skin The histamine contents of the tissues can, however, differ from time to time (see control groups in table 1 and 2) There were about 12 weeks between the two series of experiments, and it is therefore possible that the histamine content of the tissues of the rat undergoes seasonal variation

Compound 48/80 and polymyxin B were found to release 70–80% of the histamine in the skin of rats, whereas the histamine content of the brain was unaltered (table 1) These results are in agreement from the experiments of PARRATT & WEST (1957) with rats SWIFT (1956) emphasized that polymyxin B does not diffuse from the blood into the brain According to RILEY & WEST (1955), the mast cells will escape the action of compound 48/80 if they lie in the perineurium of the peripheral nerves

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From the Department of Pharmacology, University of Copenhagen
(Professor Knud O Møller, M D)

Morphine and Normorphine in the Brains of Rats given identically analgaesic Doses of Morphine, Codeine or Normorphine*

By

Torkell Johannesson and Jens Schou

(Received July 25 1963)

The polarographic method for quantitative determination of morphine in biological samples, described by PÆRREGAARD (1957) and later modified by MILTHERS (1958 & 1959) has recently been improved by JÓHANNESSON & MILTHERS (1963) so that amounts down to 0 4 µg morphine can be measured in tissue samples Normorphine can also be measured by the technique

In preliminary experiments we demonstrated morphine in the brains of rats injected with codeine in toxic amounts This gave some support to the hypothesis of SANFILIPPO (1948), who postulated that the analgaesic effect of codeine could be due to its transformation into morphine *in vivo* That O-demethylation of codeine into morphine occurs has been verified by several investigators, who have shown morphine in the urine of human subjects and of various animals given codeine However, it has been doubted whether the liberated morphine can gain access to the central nervous system (WAY & ADLER 1960), especially when given to laboratory animals in analgaesic doses (MILLER & ELLIOTT 1955) To test the validity of SANFILIPPO's (1948) hypothesis, we decided to determine and compare the analgaesic actions of morphine and codeine in rats Then, at defined levels of analgaesic effect, obtained by giving either morphine or codeine, the concentration of morphine would be determined in the brains of the animals

BECKETT *et al* (1956) put forward the hypothesis that morphine undergoes N-demethylation into *normorphine* before exerting its analgaesic action MILTHERS (1962) found that N-demethylation of morphine may occur in the brains of rats Further, JÓHANNESSON & MILTHERS (1962) concluded that the brain concentrations of morphine and normorphine

* A preliminary report of this investigation was given at the Second International Pharmacological Meeting Praha August 1963

3 Morphine did not reduce the histamine contents of brain or skin whether it was administered repeatedly or as a single injection

4 In the mast cell suspension, morphine was found to be a 300–400 times weaker liberator of histamine than compound 48/80 or polymyxin B

5 It is concluded that only at extremely high concentrations may morphine release histamine The results indicate that neither the development of morphine tolerance nor the analgaesic action of morphine is connected with liberation of histamine

Acknowledgements

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REFERENCES

Adam H M Histamine in the central nervous system and hypophysis of the dog *Regional neurochemistry*, ed by Kety and Elkes Pergamon Press 1961, pp 293–306

Feldberg W & W D M Paton Release of histamine from skin and muscle in the cat by opium alkaloids and other histamine liberators *J Physiol* 1951 114 490–509

Jóhannesson T Morphine as an inhibitor of brain cholinesterases in morphine tolerant and non tolerant rats *Acta pharmacol et toxicol* 1962 19 23 35

Jóhannesson T & J SCHOU The analgetic activity and brain concentration of morphine in tolerant and non tolerant rats given morphine alone or in combination with neostigmine *Acta pharmacol et toxicol* 1963a in press

Jóhannesson T & J Schou Morphine and normorphine in the brain of rats given identically algaesic doses of morphine codeine or nor [illegible] 1963b in press

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[illegible] ctivity *Histamine* (CIBA Foundation Symposium) Churchill London 1956 (pp 74–91)

Nasmyth P A & H C Stewart The release of histamine by opium alkaloids. *J Physiol* 1950 111 19 P

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From the Department of Pharmacology, University of Copenhagen
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Morphine and Normorphine in the Brains of Rats given identically analgaesic Doses of Morphine, Codeine or Normorphine*

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The polarographic method for quantitative determination of morphine in biological samples, described by PÆRREGAARD (1957) and later modified by MILTHERS (1958 & 1959), has recently been improved by JÓHANNESSON & MILTHERS (1963) so that amounts down to 0 4 µg morphine can be measured in tissue samples Normorphine can also be measured by the technique

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g[illegible] orphine before exerting its analgaesic action MILTHERS (1962) found that N-demethylation of morphine may occur in the brains of rats. Further, JÓHANNESSON & MILTHERS (1962) concluded that the brain concentrations of morphine and normorphine

* A preliminary report of this investigation was given at the Second International Pharmacological Meeting Praha August 1963

might be nearly the same if given in identically analgaesic doses to rats This assumption was, however, not tested experimentally We therefore included normorphine in our experiments and compared its analgaesic effect with those of codeine and morphine We also determined nor morphine in the brains of rats given this drug in amounts that produced the same levels of analgaesia as those in the experiments with morphine and codeine

Methods

Adult male albino rats of a single strain (140 230 g) were maintained under constant environmental conditions with free access to water and a commercial food preparation before the experimental period

Analgaesimetry Electrical stimulation was used as the pain stimulus Impulses of square wave type were delivered continously from an impulse generator (Kaiser ® stimulator) at a frequency of 1 Hertz Each stimulus had a strength of 3 Volts and lasted for 20 milliseconds The voltage of the stimuli was checked continuously by a cathode ray oscillograph inserted in the circuit The electrodes were made of two injection needles (2R2 No 16) attached in a Perspex holder 8 mm apart and with [illegible] key

[illegible]

4 cm from the hairy margin of the tail root The rat was then allowed to move [illegible] in an open plastic dish measuring 35×32×12 cm When the animal was completely quiet after the insertion of the electrodes the circuit was closed by pressing the telegraph key Only a squeak was regarded as a positive pain reaction Under the described standardised conditions about 80% of the animals did squeak at the first second third or fourth stimulus generally reacting to the first two stimuli Only those animals (reactive rats) were used for the further experiments the rest being discarded Of a group of such reactive rats the vast majority did indeed squeak at the first or second stimulus on both occasions if the reactive rats were tested twice at an interval of 30–45 minutes The number of stimuli needed to provoke a squeak did not vary by more than ±2

In the experiments with the analgaesics all the animals were tested within 20 minutes before administration of the drug The drug was injected and the animals were tested again 30 minutes later We defined analgaesia as occurring when 3 sti muli more than the number of stimuli given to provoke a squeak in the control test could be given without response

Injection of analgaesics The drugs were dissolved in saline (0 9%) and injected intraperitoneally by means of a tuberculin syringe divided to deliver 0 01 ml The concentrations of the solutions were adjusted so that the injected volumes always amounted to 2 ml/kg

Analytical procedures

Determinations of morphine in the brains were performed as described by JÓHANNESSON & MILTHERS (1963) The procedure includes descending paper chroma tography for 16 hours The average recovery for amounts of 0 5–1 0 μg morphine

... that can be measured quantitatively are about ... to as ... ession ... able amounts" (*n d*) ...

shown on the polarogram.

Normorphine was extracted from the brain as described by MILTHERS (1961) Paper chromatography was extended to 36 hours to secure sufficient separation Normorphine was then eluated and determined by the same technique as morphine The average recovery was about 65% for 1 µg normorphine In calculating the concentrations of normorphine allowance was made for the low percentage recovery

The results are expressed as µg of the chloride per g brain and refer to "free" morphine or normorphine (autoclaving omitted) The results of the quantitative analysis are given as decimal fractions When these figures include two decimals the first one is significant, the second being indicative only For *statistical evaluation* we employed Student's t test. In these calculations *n m*, referring to a perceptible morphine reaction on the polarogram, but one that could not be measured quantitatively, is entered as 0 10 µg/g, and *n d* is entered as 0, to signify that no trace of morphine reaction could be seen in the polarogram

Results

Preliminary experiments were performed to establish the best time after the injection of drugs for analgaesimetry and removal of the brain for analysis Further, the range of concentration of morphine in the brain was studied after administering morphine and codeine in larger doses The results (table 1 and 2) indicate that the highest concentration of morphine was found approximately 30 minutes after intraperitoneal injection of morphine and codeine This time period was always used in the subsequent experiments

Table 1

Codeine dose (mg/kg)	75				150	200
Survival time (minutes)	15	30	45	60	30	30
	n.d 0 20 0 26	0 39 0 44 0 45	0 24 0 31 0 42	*n m* *n m* 2.2	0 36 0 62 1 0	1 5 1 6 3 8

Table 2

The concentration of morphine in brains from rats (µg/g) injected intraperitoneally with 20 or 50 mg/kg and killed at various times after injection

Morphine dose (mg/kg)	20				50
Survival time (minutes)	15	30	45	60	30
	0 40	0 42	0 38	n m	2 0
	0 46	0 81	0 61	n m	2 1
	1 1	1 2	–	n m	2 2
	–	1 7	–	–	3 0
	–	–	–	–	3 9

Analgaesimetry

9 groups of rats were used, each of 30–50 animals, allowing 3 dosage levels of each drug to be examined Morphine was used at doses of 2 5 5 0 and 10 mg/kg, codeine and normorphine were given in amounts of 10, 20 and 40 mg/kg The results are summarized in fig 1 The regression coefficients do not significantly differ from each other The potency factors for normorphine, codeine and morphine were approximately 1 1 8 5 8 The relative potency of morphine and codeine was 3 3 1

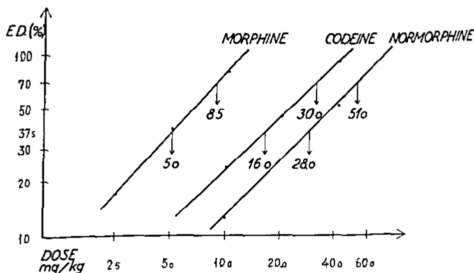


Fig 1 The analgaesic effect of morphine codeine and normorphine in rats The drugs were given by intraperitoneal injections and the analgaesic effect was determined 30 minutes later [illegible] mg kg (log scale) [illegible] ive analgaesic response [illegible] ses of morphine codeine [illegible] 37 5 % and 70% of the and normorphine that we [illegible] animals

Morphine and normorphine analyses

From the dose response curves we then chose doses of the three drugs that produced analgaesia in 37 5 and 70% of the animals These doses were, for morphine 5 and 8 5 mg/kg, for codeine 16 and 30 mg/kg and for normorphine 28 and 51 mg/kg respectively In further experiments these doses were given to groups of rats varying in number from 8 to 20 After measurement of their sensitivity to electric stimuli, the drug was given and the analgaesic response was tested 30 minutes later Immediately after the rats were killed, and the brains removed for chemical analyses The results of these experiments are summarized in table 3 and 4 In several of the analyses only trace amounts were demonstrated and could not be evaluated quantitatively These analyses are given as *n m* in the tables, two samples in which no morphine could be detected are given as *n d* The analgaesimetric responses determined did not differ significantly (P > 0 05) from the expected values (37 5 and 70%)

For determining the mean concentrations and standard errors of the means in the different experimental groups 0 10 µg/g was used as the value for those given as *n m* in the tables In this way the mean concentrations of morphine in the brains of codeine treated rats amounted to 0 19 ± 0 03 µg/g at the 37 5% analgaesic level No statistical difference existed between this figure and the mean concentration for the animals treated with morphine to obtain an identical degree of analgaesia (P > 0 1) This figure was 0 26 ± 0 07 µg/g At the 70% analgaesic level codeine

Table 3

Concentrations of morphine (µg/g) in the brains of rats given identically analgaesic [illegible] analysis

A animals showing analgaesia
NA animals showing no analgaesia

	37 5% analgaesia		70% analgaesia	
	NA	A	NA	A
Morphine given	0 23 (2)	0 21 (2)	0 24 (2)	0 24 (2)
	0 36 (2)	0 24 (2)	0 32 (2)	0 26 (2)
	0 54 (2)	0 52 (2)	*n m* (2)	0 30 (1)
	n d (2)	*n m* (2)	*n m* (2)	0 36 (2)
	–	–	*n m* (1)	*n m* (2)
	–	–	–	*n m* (2)
Codeine given	0 20 (2)	0 21 (2)	0 31 (1)	0 28 (2)
	0 24 (2)	*n m* (2)	*n m* (2)	0 32 (2)
	0 27 (2)	–	–	0 34 (2)
	n m (2)	–	–	*n m* (1)

Table 2.

The concentration of morphine in brains from rats (μg/g) injected intraperitoneally with 20 or 50 mg/kg and killed at various times after injection

Morphine dose (mg/kg)	20				50
Survival time (minutes)	*15*	*30*	*45*	*60*	*30*
	0 40	0 42	0 38	*n m*	2 0
	0 46	0 81	0 61	*n m*	2.1
	1.1	1 2	–	*n m*	2 2
	–	1.7	–	–	3 0
	–	–	–	–	3 9

Analgaesimetry.

9 groups of rats were used, each of 30–50 animals, allowing 3 dosage levels of each drug to be examined. Morphine was used at doses of 2 5, 5 0 and 10 mg/kg; codeine and normorphine were given in amounts of 10, 20 and 40 mg/kg The results are summarized in fig. 1. The regression coefficients do not significantly differ from each other. The potency factors for normorphine, codeine and morphine were approximately 1:1.8:5.8 The relative potency of morphine and codeine was 3.3.1.

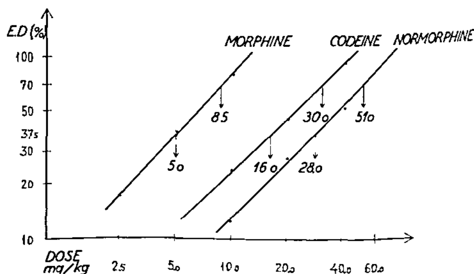


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Concentrations of morphine (μg/g) in the brains of rats given identically analgaesic [illegible] analysis.

A animals showing analgaesia
NA animals showing no analgaesia

	37 5% analgaesia		70% analgaesia	
	NA	A	NA	A
Morphine given	0 23 (2)	0 21 (2)	0 24 (2)	0 24 (2)
	0 36 (2)	0 24 (2)	0 32 (2)	0 26 (2)
	0 54 (2)	0 52 (2)	*n m* (2)	0 30 (1)
	n d (2)	*n m* (2)	*n m* (2)	0 36 (2)
	–	–	*n m* (1)	*n m* (2)
	–	–	–	*n m* (2)
Codeine given	0 20 (2)	0 21 (2)	0 31 (1)	0 28 (2)
	0 24 (2)	*n m* (2)	*n m* (2)	0 32 (2)
	0 27 (2)	–	–	0 34 (2)
	n m (2)	–	–	*n m* (1)

Table 4

[illegible]

used for each analysis
A animals showing analgaesia
NA animals not showing analgaesia

	37 5% analgaesia		70% analgaesia	
	NA	A	NA	A
Normorphine given	0 59 (2)	0 83 (2)	1 0 (1)	1 2 (1)
	0 62 (2)	0 85 (2)	1 4 (1)	1 2 (1)
	0 69 (2)	–	–	1 2 (1)
	0 73 (2)	–	–	1 4 (1)
	–	–	–	1 5 (1)
	–	–	–	1 5 (1)
Mean concentration	0 72 ± 0 04 µg/g		1 3 ± 0 1 µg/g	

administration produced a mean concentration of morphine in the brain amounting to 0 24 ± 0 05 µg/g, the figure after administration of mor phine being 0 20 ± 0 04 µg/g These figures also do not differ significantly ($P > 0\ 5$) However, no tendency to a higher brain concentration could be demonstrated in the animals submitted to 70% analgaesia than in those submitted to 37 5% We do not place much emphasis on this fact because of the two very high concentration found in the 37 5% morphine analgaesia group (see table 3) If these figures are excluded from the mean calculations, this group would show a mean of only 0 19 µg/g which may be a more valid figure The same mean is found if all the results from the 37 5% analgaesia group (obtained with morphine and codeine) are bulked, whereas the mean for the 70% analgaesia group is 0 22 µg/g calculated in this way

Discussion

In several experimental animals and human subjects morphine has been demonstrated in the urine after codeine administration This was first shown in the rat by ADLER & LATHAM (1950), and later confirmed by several groups of workers in experiments on different species (ADLER *et al* 1955, WOODS *et al* 1956, PÆRREGAARD 1958, a review is given by WAY & ADLER 1962) *In vitro* the demethylation of codeine to morphine was demonstrated by AXELROD (1955), who incubated liver micro somes with codeine in the presence of reduced triphosphopyridine nucleotide and oxygen

The analytical evidence for *in vivo* transformation of codeine into morphine was, as mentioned above, not obtained until 1950 However,

already in 1938 WOLFF had suggested that the reason why morphine addicts could be maintained on codeine was most likely because such individuals could demethylate codeine and thereby liberate morphine Later SANFILIPPO (1948) postulated that the analgaesic action of codeine is due to its conversion into morphine In our experiments the concentration of morphine in the brain of rats given either codeine or morphine in order to obtain the same degree of analgaesia was found to be within the same range (table 3) These results strongly support the view that codeine acts after transformation into morphine Nevertheless, objections can be raised to this conclusion The sensitivity of the analytical procedure is not sufficient to determine the concentration of morphine present in all the materials Most of the measurements were performed on amounts around the limits of the method for quantitative determination Further, most of the analyses were performed on bulked brains from two animals, moreover it is possibly erroneous to try to correlate total brain concentration and analgaesic activity of a drug *However, we feel justified in concluding that morphine is found in the brain after administration of codeine in analgaesic doses to rats* The concentrations were in fact at the same quantitative level as if morphine had been given to obtain the same degree of analgaesia

We also attempted to obtain evidence about the hypothesis relating N demethylation of morphine to analgaesia (BECKETT *et al* 1956) MILTHERS (1962) has recently shown normorphine in the brain of hepatectomized rats injected with morphine One of the predictions of the hypothesis is that morphine and normorphine are drugs of almost equal analgaesic potency However, several investigators have found normorphine to be less active than morphine (e g. MILLER & ANDERSON 1954, LASSAGNA & DE KORNFELD 1958) JÓHANNESSON & MILTHERS (1962) suggested that the lower analgaesic effect of normorphine might be explained by its lower degree of penetration into the brain They assumed, however, that morphine and normorphine are found in similar amounts in the brains of rats given identically analgaesic amounts of the two drugs

In our analgaesimetric experiments morphine was found to be 6 times as potent as normorphine Further, the concentrations of normorphine found in the brains from animals given this drug were much higher than those of morphine in animals given morphine in identically analgaesic doses Although these results cannot be regarded as conclusive, because whole brains were used for the analyses, the hypothesis of BECKETT *et al* is strongly contra indicated by our findings

Several investigators have compared the analgaesic potencies of codeine and morphine in different species This ratio of analgaesic potencies has

been found to range from 1 2 to 1 10 (SCHAUMANN 1957), the same ratio for normorphine and morphine ranging from 1 2 to 1 18 RAUSCH *et al* (1959) compared the analgaesic potency of the three drugs in mice and found morphine to be 7 and 12 times more potent than codeine and normorphine, respectively From our determinations on rats, morphine is 3 3 times as potent as codeine, and the ratio between the analgaesic effects of morphine and normorphine is 6

The animals whose brains were removed and analysed for morphine or normorphine were all tested analgaesimetrically immediately before being killed In table 3 and 4 the analytical results are divided into those from rats showing analgaesic and those showing non analgaesic reactions These results, even though few in number, indicate that the analgaesic effect in the rat is independent of the total amount of morphine or normorphine found in the whole brain

Our results are, as already mentioned, indicative rather than conclusive We believe that a greater sensitivity of the analytical procedure would make possible a more appropriate quantitative comparison between biological activity and concentration of analgaesics in different parts of the central nervous system than we could achieve in our investigation

Summary

An analgaesimetric procedure on rats was employed to compare the analgaesic potencies of morphine, codeine and normorphine Morphine was found to be 3 3 and 6 times more potent than codeine and normorphine, respectively

From the dose-response curve identically analgaesic doses of the three drugs were choosen that would be expected to produce 37 5 and 70% analgaesia At these dose levels the concentrations of morphine in the brain were in the same range whether analgaesia was produced by morphine or codeine (0 19–0 24 µg/g on the average) These results are in agreement with the assumption that codeine is O demethylated into morphine before exerting its analgaesic effect

The concentration of normorphine in the brain was 4–5 times higher than the concentration of morphine when these drugs were administered in identially analgaesic doses This invalidates the hypothesis that morphine analgaesia is due to normorphine

Acknowledgements

This paper is dedicated to Mr Johannes Nielsen (Kobenhavn) One of the authors (T J) received stipends from *Visindasjóður* (Reykjavik)

and from *Fonden til Lægevidenskabens Fremme* (København) We thank Miss Karin Dyhrfjeld for valuable technical assistance during the performance of the experimental work.

REFERENCES

Beckett, A H, A F Casy & N J Harper Analgesics and antagonists Some steric and chemical considerations, Part III The influence of the basic group on the biological response *J Pharm Pharmacol* 1956, 8, 874–883

Jóhannesson, T & K Milthers Morphine and normorphine in the brain of rats A comparison of subcutaneous intraperitoneal and intravenous administration *Acta pharmacol et toxicol* 1962, 19, 241–246

Jóhannesson, T & K Milthers Determination of morphine *Radiometer*

Mill

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Milt

Pærregaard, P A new method for quantitative determination of small amounts of morphine in human urine *Acta pharmacol et toxicol* 1957, 14 38–52

Pærregaard, P The liberation of morphine from

Sanfilippo G Contributo sperimentale all' ipotesi della smetilazione della codeina nell organismo I Influenze della dose sull' assuefazione alla codeina II Assuefazione alla codeina attenuta con somministrazione prolungata di morfina *Boll Soc Ital Biol sper* 1948, 24, 723–726

Schaumann, O Morphin und Morphinähnlich wirkende Verbindungen *Handbuch der experimentellen Pharmakologie*, vol 12, Springer, Berlin 1957, (p 111)

Way, E Leong & T K Adler The biological disposition of morphine and its surrogates 2 *Bull Wld Hlth Org* 1962, 26, 51–66

Way, E Leong & T K Adler The pharmacologic implications of the fate of morphine and its surrogates *Pharmacol Rev* 1960, 12, 383–446

Wolff, P The significance of codeine as a habit forming drug *Quart Bull League Nat Health Org* 1938, 7, 546–580

Woods, L A, H E Muehlenbeck & L B Mellett Plasma levels and excretion of codeine and metabolites in the dog and monkey *J Pharmacol* 1956, 117, 117–125

Acta pharmacol et toxicol 1963, 20, 174–180

From the Department of Pharmacology and Toxicology,
Royal Veterinary and Agricultural College, Copenhagen

Spontaneous and Pralidoxime-induced Re-activation of Brain Cholinesterase in the Chicken after Fatal Nitrostigmine (Parathion) Poisoning

By

O. Karlog and F. Poulsen

(Received July 24 1963)

An increase in poisoning of poultry by accidents with or misuse of organo-phosphorus insecticides has emphasized the problems of verification By the screening method of FRIEDBERG & SAKAI (1958) the probability of poisoning with organo phosphorus compounds can be assessed by the degree of re-activation of inhibited blood and brain cholinesterase obtained during incubation with pralidoxime This method was considered suitable for the purposes of the study reported here In chickens the cholinesterase activity of the blood is confined to the plasma (AUGUSTINSSON 1948, BLABER & CUTHBERT 1962), and the hematocrit cannot be determined on the dead animal Preliminary investigations showed the brain tissue to be a more suitable test material Before the routine use of this screening method, it was found necessary to determine the cholinesterase activity in brain tissue from normal chickens at different times after death and to investigate possible spontaneous re activation (ALDRIDGE 1953, DAVISON 1953a, BLABER & CREASY 1960a & b, JENSEN-HOLM 1960) in poisoned chickens The effect of the age of the carcass material on spontaneous and induced re-activation was also investigated (HOBBIGER 1955, FRIEDBERG & SAKAI 1958)

As the insecticide nitrostigmine*) is the most frequently used organo phosphorus compound, the experiments were performed on chickens after fatal poisoning by its oral application

To imitate the unfavorable conditions found in practice, the dead animals were stored at 18–20°C Besides the cholinesterase determinations, chemical determinations of nitrostigmine and its metabolites were performed on the contents of the crop, the gizzard and the intestines

*) Nitrostigmine (NFN) parathion = 0 0 diethyl 0 p nitrophenyl thiophosphate

Material and Methods

Twenty two chickens (weight 1 2-1 6 kg) were used for the experiments, twelve were used to determine levels of brain cholinesterase, and the remaining ten were dosed by stomach tube with 35 mg nitrostigmine (Bladan F ®, Agro-kemi Ltd, Copenhagen) per kg body weight Analyses of the nitrostigmine preparation by paper and thin layer chromatography showed only traces of p-nitrophenol and no para-oxon or isomeric nitrostigmine

After death the material from two of these animals was immediately analyzed, the remaining carcasses being stored undissected at 18-20°C for 1, 2, 4 and 8 days After removal of the brain for cholinesterase determination, the gut was taken out whole and emptied The gut contents were then analyzed for nitrostigmine and metabolites by the method of KARLOG (1957)

The cholinesterase activity of the brain tissue was measured by the Warburg technique (AUGUSTINSSON 1948) The cerebral hemispheres cooled in icewater were homogenized in 1 ml of bicarbonate buffer for 2 minutes at maximal speed in an MSE 7700-B-homogenizer The re-activation was accomplished by incubation with 10^{-3}M pralidoxime iodide in Warburg buffer (pH 7 6) for 2 hours at 20-22°C The cholinesterase activity was recorded as the output of CO_2 per 100 mg of brain tissue per 30 min

Results

In table 1 are shown the results of the cholinesterase determinations in brain tissue from the normal chickens stored under the same conditions as the poisoned animals In the fresh material a uniform level of activity was found but after storage for four days there was a 15-25% decrease in activity No further decrease resulted on storage for eight days The addition of pralidoxime did not alter activity in either the fresh or the stored material

The chickens poisoned by oral application of nitrostigmine developed muscular tremor, dyspnoea, paresis, increased glandular secretion, diarrhoea terminal convulsions and coma Death occurred 18-50 minutes after the application In table 2 the results of the cholinesterase determinations show that the activity was low in the brain tissue from the chickens examined immediately after death In chickens stored for 1 or 2 days the enzyme activity was at least twice as high but still considerably lower than in the control normal birds After 4 days storage the activity was four times higher than immediately after death

Compared with the control chickens, an inhibition of some 60% was found

After storage for 8 days the activity was six to eight times higher than that immediately after death, comparison with the unpoisoned controls showed only 30% inhibition

It can be seen that there is a large spontaneous re activation of inhibited

Table 1

Brain cholinesterase activity in 12 normal chickens before and after incubation with pralidoxime iodide

Number of days between death and analysis	Chick No	Brain cholinesterase activity		
		µl CO_2/100 mg/30 min	After incubation with 10^{-3}M pralidoxime iodide µl CO_2/100 mg/30 min	activity before incubation with pralidoxime activity after incubation with pralidoxime
0	1	1400	1280	1 09
	2	1380	1290	1 07
	3	1260	1200	1 05
	4	1420	1290	1 10
4	5	1050	1150	0 91
	6	1060	1100	0 96
	7	950	1040	0 91
	8	1050	1080	0 97
8	9	1110	1150	0 97
	10	1100	1090	1 01
	11	980	1000	0 98
	12	1040	1080	0 96

Table 2

Brain cholinesterase activity in 12 chickens dead after poisoning with nitrostigmine the activity measured before and after re-activation with pralidoxime

Number of days between death and analysis	Chick No	Brain cholinesterase activity		
		µl CO_2/100 mg/30 min	After incubation with 10^{-3}M pralidoxime iodide µl CO_2/100 mg/30 min	activity before incubation with pralidoxime/activity after incubation with pralidoxime
0	13	10	1120	0 09
	14	120	1280	0 09
1	15	285	1120	0 25
	16	435	780	0 56
2	17	235	900	0 26
	18	315	950	0 33
4	19	430	840	0 51
	20	420	670	0 63
8	21	635	800	0 79
	22	800	980	0 82

cholinesterase during storage of the carcass at 18–20°C, especially during storage for more than 4 days

Table 2 also shows that inhibited cholinesterase incubated with pralidoxime immediately after death could be completely re activated to the level present in normal chickens After 1–4 days of storage a considerable re activation could still be produced, but after storage for 8 days no increase in activity from addition of pralidoxime could be shown

The results of the nitrostigmine determinations are shown in table 3 It can be seen that 32 to 53% of the administered dose was recovered with no relationship between recovery and duration of storage Analyses by paper chromatography and spectrophotometry showed that only 1–2% of the nitrostigmine was hydrolysed to p-nitrophenol. No para-oxon was detected

Discussion

The cholinesterase activity found in the cerebrum of fresh normal chickens was of the same order as that found by DAVISON (1953b), and the observed small influence of storage on the activity is in good agreement

Table 3

Nitrostigmine in contents of crop gizzard and intestines of 10 chickens after fatal poisoning with nitrostigmine

Number of days between death and analysis	Chick No	Nitrostigmine administered mg	Nitrostigmine recovered from the contents of crop gizzard and intestine mg	Recovery per cent
0	13	49	25 8	53
	14	56	20 7	37
1	15	49	20 6	42
	16	42	16 6	40
2	17	49	15 9	32
	18	46	15 6	34
4	19	49	18 3	39
	20	42	16 4	39
8	21	42	21 2	50
	22	49	16 5	34

with the results of FRIEDBERG & SAKAI (1958) on material from mammals and man

The percentage of the applied nitrostigmine recovered from the gut of the poisoned chickens, 32–53%, suggest that there was the same degree of absorption of this compound by all the treated animals Therefore inhibition of the brain cholinesterase immediately after death was probably about 90% in all of them, with gradual re activation during storage This spontaneous re activation, which is probably caused by hydrolysis of the phosphorylated enzyme (ALDRIDGE 1954), is highly dependent on temperature (FRIEDBERG & SAKAI 1958, JENSEN HOLM 1960) Our results on spontaneous re activation during an 8 day period agree well with those obtained by FRIEDBERG & SAKAI (1958)

Storage of the carcase of a poisoned animal under unfavourable conditions may result in the spontaneous re-activation of cholinesterase to normal levels within one week This has as much effect on the practical value of the method for investigating nitrostigmine poisoning as has the effect of possible transphosphorylation on the re activatability of the cholinesterase (HOBBIGER 1955 & 1957, WILSON 1955, DAVIES & GREEN 1956) In our study nitrostigmine poisoning did not seem to produce this state of completely irreversible cholinesterase inhibition

FRIEDBERG & SAKAI (1958) in their paper, put forward a re activation index

$$\frac{\text{Cholinesterase activity without pralidoxime}}{\text{Cholinesterase activity with pralidoxime}}$$

and stated that a value for this index of one or less gives evidence of an inhibition of cholinesterase caused by organophosphorus insecticides They also found an index of approximately 1 2 for cholinesterase in tissue from non poisoned animals In our study the index was about 1 0 for such material This difference is probably due to the fact that the preparation of pralidoxime used by us showed little effect on normal cholinesterase activity, whereas the preparation used by FRIEDBERG & SAKAI produced an inhibition of about 20% It can thus be seen from our studies on chickens that an index of 0 8 or less gives a good indication [illegible]

index of 0 8 or more the possibility of poisoning with organophosphorus compounds cannot be excluded, especially if the material has been stored for some time Poisoning with nitrostigmine could still be verified by a chemical analysis of gastro intestinal contents as 30–50% of the administered dose can be detected after storage at 18–20°C, for 8 days

Summary.

Oral administration of 35 mg of nitrostigmine per kg body weight to ten chickens resulted in acute fatal poisoning The cholinesterase activity of their brain tissue was examined after storing the carcasses at 18–20°C for 0 1, 2, 4 and 8 days Immediately after death the activity was lowered to about 10% that of normal chickens, storage, resulted in a considerable spontaneous re activation, the amount depending on its duration After storage for 4 days the activity was about 40% and after 8 days about 70% of the activity in brain tissue from normal chickens stored under the same conditions On incubation with 10^{3} M pralidoxime iodide for 1 hour at 20–22°C the inhibited brain cholinesterase could be completely re activated immediately after death After storage for 1–4 days it was still possible to obtain considerable re activation, whereas in brain tissue stored for more than 4 days artificial re activation was difficult to recognize because of the spontaneous re activation that had taken place

Acknowledgement

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REFERENCES

Aldridge W N The inhibition of erythrocyte cholinesterase by tri-esters of phosphoric acid 3 The nature of the inhibitory process *Biochem J* 1953 54 442–448

Aldridge W N Anticholinesterases Inhibition of chol nesterase by organo-phosphorus compounds and reversal of this reaction Mechanisms involved *Chem & Ind* (*London*) 1954 473–476

Augu[illegible] ative enzymology

Blabe[illegible] of cholinesterase Blood cholineste

Blabe[illegible] of cholinesterase

Blab[illegible]

Davies D R & A L Green The [illegible]

Friedberg, K D & F Sakai Spezifischer Nachweis von Vergiftungen mit Alkylphosphaten (E 600, E 605, Systox) in Blut und Hirngewebe mit

phosphato group Brit J Pharmacol 1955, 10, 356-362

with a potent cholinesterase inhibitor (paroxan) *Acta pharmacol et toxicol* 1960, 17, 255-266

Discuss Faraday Soc 1955, 20, 119-134

Acta pharmacol et toxicol 1963, 20 181-185

From the Department of Anaesthesia (Heads E Waino Andersen S H Johansen and V Dyrberg) and the Cardio-respiratory Laboratory (Head P Eskildsen) Copenhagen County Hospital in Gentofte Hellerup Denmark

The Effect of Pirexyl ® on Normal and Depressed Respiration

By

S H Johansen, Mogens Jørgensen and V. Dyrberg

(Received June 17 1963)

Respiratory depression is an undesirable side effect of those antitussive agents that act by raising the central threshold for cough If the cough reflex can be interrupted on its afferent limb or even more peripherally in the reflexogenic zones of the tracheobronchial tree, cough suppression is not accompanied by medullary depression (H A BICKERMAN 1962) With the object of developing an antitussive agent having minimal central action 1 (2 benzyl phenoxy) 2 piperidinopropane phosphate (pirexyl ®) was chosen from a series of substituted benzyl phenoxy propanes and tested on man (RUBINSTEIN *et al* 1962), cough suppression without serious side effects was reported

In animal experiments pirexyl ® was shown to have an inhibiting effect on the induced cough in cats as well as rabbits and dogs It appeared that the agent exerted its antitussive effect both centrally and at the periphery, but that the central depressant effect was observed only after larger doses In cats and rabbits, pirexyl ® in doses of 1-10 mg/kg would increase ventilation and *in rabbits* the respiratory depressant effect of morphine was counteracted by 5-10 mg of pirexyl ® Prompted by the above observations, an *investigation* into the respiratory effect of pirexyl ® in man has been carried out

Method

The experiments were performed on three normal men Table 1 shows their ages and body measurements The method for recording respiratory function has been described in details elsewhere (DYRBERG *et al* 1962) The subjects were made to breathe into a closed circuit with a reservoir bag filled before each experiment with 10 l of pure oxygen, and the expired carbon dioxide was allowed to accumulate in

Table 1.

Characteristics of the three experimental subjects

Subjects	Age in years	Height in cm	Weight in kg
O T	20	177,5	77 5
N M	23	173,5	62 5
H I	20	185,5	74 0

the circuit during the experiments End-expiratory carbon dioxide was continuously measured on an infrared analyser (Capnograph, Godart) and the expired volume with a pneumotachograph (Fleisch) Both variables were continuously recorded on a Honeywell ® ultraviolet recorder

Each experiment consisted of 13 rebreathing procedures, during which endogenous carbon dioxide was allowed to accumulate in the breathing circuit until a concentration of 8 per cent was reached, when the procedure was ended A rebreathing procedure would last from 6 to 7 minutes and was repeated every 10–15 minutes The first 5 rebreathing procedures served as controls The subsequent 8 procedures were done under influence of the drugs tested The control curves were recorded within the first 75 minutes, and the subsequent period of observation of the drug effect in each experiment amounted to about 2 hours On each subject two experiments were performed on separate days

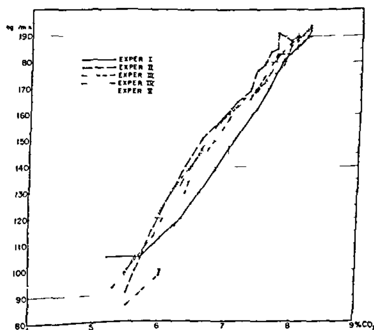


Fig 1 Response in respiratory minute volume (l/min) to increasing concentrations of carbon dioxide (% CO_2) in expired air (semilogarithmic plotting) 5 control curves for subject N M

I The respiratory effect of pirexyl ®, 10 mg per 70 kg body weight, was tested alone

II The respiratory effect of pethidine, 50 mg per 70 kg body weight, was tested during 3 rebreathing procedures, and then pirexyl ®, 10 mg per 70 kg body weight, was injected and the effect on the respiratory response was observed during 5 rebreathing procedures

All injections were given intravenously

From the continuous record of respiratory minute volume and CO_2 per cent in the end-expiratory air, concomitant values were tabulated Under conditions of repeatedly changing pCO_2 levels, linearity of ventilation against pCO_2 cannot be obtained (BELVILLE *et al* 1960) Consequently the logarithm of the ventilation was plotted against pCO_2 (fig 1) In each experiment all the values of CO_2 per cent and of log ventilation from the first 5 control procedures were pooled, as were all values obtained under influence of the drugs tested, and the different regression equations were determined. Ventilation in litres per minute at 7 per cent CO_2 was calculated from the equations and used as indicator of the ventilatory response in the experiments A CO_2 per cent of 7 was chosen because changes in the sensitivity of the respiratory centre stand out more clearly at elevated tensions of carbon dioxide (ECKENHOFF & OECH *1960)*

Results

The respiratory effects of pirexyl ® alone and along with pethidine were evaluated from the calculated ventilatory response to 7 per cent CO_2 The results are presented in fig 2

Pirexyl ® The respiratory effect of 10 mg pirexyl ® per 70 kg body weight was small and inconsistent, giving rise to a slight increase in respiratory minute volume in two subjects, and an equally slight decrease in the third

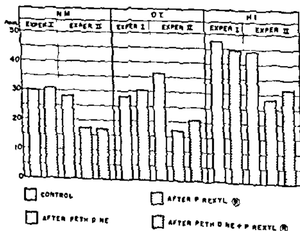


Fig 2. Graphic representation of the calculated respiratory minute volume (l/min.) at 7% CO_2 in end-expiratory air after injection of pirexyl ® (10 mg/70 kg) or of pethidine (50 mg/70 kg) and then pirexyl ® 10 mg/70 kg.

Pethidine and then pirexyl ® In all subjects 50 mg pethidine per 70 kg body weight produced a decrease in ventilation of between 30 and 50 per cent

When pirexyl ®, 10 mg per 70 kg body weight, was injected in the presence of pethidine, the ventilatory responses were not pronounced, although in two subjects increases in ventilation were observed In each the respiratory minute volume was far from being restored to the control values, that is, the ventilatory responses before injection of pethidine In the third subject no alteration in ventilation occurred

No side effects were observed after the intravenous injection of this dose of pirexyl ®

Discussion

Pethidine exerts a depressant effect on the respiratory centre leading to diminished ventilation, mainly through a reduction in tidal volume The demonstration of this effect, after a dose of 50 mg per 70 kg body weight supports the contention of ECKENHOFF & OECH (1960) that the rebreathing technique reveals drug-induced alterations in ventilation It has been demonstrated by our experiments, that pirexyl ® in the dose given exerts no depressant effect on the respiratory centre

The question whether pirexyl ® in man has respiratory stimulatory properties is less easy to answer Small increases in ventilation were admittedly observed in two subjects when the drug was given alone, and also when it was given after pethidine, but in the latter instance the ventilatory increase was not of an extent that would in any way suggest a specific and effective antagonism to a central depressant narcotic

Summary

The influence of an antitussive agent, pirexyl ®, on the ventilatory response to carbon dioxide was tested on three normal men by a rebreathing technique Neither when given alone, nor when given to persons whose respiration had been depressed with a narcotic, did pirexyl ® have any significant effect on respiration

Acknowledgement

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REFERENCES

Belv

Bick

Dyrberg V, S H Johansen, & M Jørgensen Effect of tetrahydroaminacrine (T H A) on normal and depressed respiration *Acta anaesth Scandinav* 1962, 6, 77–83

Eckenhoff, J E & S R Oech The effect of narcotics and antagonists upon respiration and circulation in man *Clin Pharmacol Therap* 1960, 1, 483–524

Rubinstein, K., J G A Pedersen, K Hermansen, H Hint, & J Opresnik Pirexyl – Et nyt hostestillende middel *U f L* 1962, 124, 175–178

Acta pharmacol et toxicol 1963, 20, 186–200

From the Pharmacological Laboratory of Dumex Ltd,
Copenhagen, Denmark

The Teratogenic Effects of Thalidomide, Imipramine HCl and Imipramine-N-Oxide HCl on White Danish Rabbits.

By

Valdemar Larsen

(Received November 8, 1963)

When suspicion began to appear in West Germany, Great Britain and Australia that there might be a connection between an increase in the number of malformed babies born and the therapeutic use of thalidomide, alpha-pthalimido glutarimide (WIEDEMANN 1961, MCBRIDE 1961, LENZ 1961, SPEIRS 1962, SMITHELLS 1962, MORGAN 1962, PLIESS 1962, LECK & MILLAR 1962, GRAINGER *et al* 1962), a drug introduced to therapy in 1956, investigation was soon begun to ascertain whether such a harmful effect could have been foretold in experiments on animals

Now we know that, if by chance the right strain of the right test animal had been used or if some special technique had been employed, a warning might have been given The drug in question may only have a teratogenic effect on certain strains of mice (GIROUD *et al* 1962, HAGEN 1963) and rabbits (SOMERS 1962, SELLER 1962) Many authors have shown it to decrease fertility in rats, but to have no teratogenic effect on them (e g CHRISTIE, 1962) However, by means of a particular technique MCCOLL *et al* (1963) have shown it to produce many skeletal malformations in rats The teratogenic effect of thalidomide can also be demonstrated on chick embryos (KEMPER 1962, EHMANN 1963, WILLIAMSON *et al* 1963) and rabbit embryo, blastocysts (LUTWAK-MANN & HAY 1962) Much work has been published on congenital malformations (KALTER & WARKANY 1959, CIBA FOUNDATION 1960) and on the teratogenic and embryotoxic effect of thalidomide (a o MELLIN & KATZENSTEIN 1962) Below there will only be mentioned a few of the authors who have found after thalidomide and other drugs effects and abnormalities, similar to those mentioned in this paper FELISATI (1963) found a hook-like position of the forepaw in rabbit foetuses, and ROBSON & SULLIVAN (1963) found

Abstract

pressed response after pethidine

LARSEN VALDEMAR (From the Pharmacological Laboratory of Dumex Ltd, Copenhagen, Denmark) *The Teratogenic Effects of Thalidomide, Imipramine HCl and Imipramine N-Oxide on White Danish Rabbits* Acta pharmacol et toxicol, 1964, 20, (3), 186–200 (4 tables, 2 figs 28 ref)

Abstract

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Abstract

The properties of a neurone blocking agent N (β-guanidinoethyl

misshapen front paws after the administration of imipramine to the mother MURPHY *et al* (1957) found abnormal forelegs and hindlegs and abnormally short tails in young rabbits after treating the mother with thiadiazole They have also shown that the same agent produced shortening of the upper beak in chick embryos EHMANN (1963) also found beak and eye abnormalities in chick embryos after thalidomide LENZ (1961) has reported aplasia of the kidneys in children after the administration of thalidomide to the mothers If nothing is stated below to the contrary, it is to be understood that the abnormalities mentioned were produced by administering thalidomide to the mother during pregnancy WILLIAMSON (1963) found both eyelid defects and failure of ventral closure in chick embryos RUSSELL & MCKICHAN (1962) found umbilical cord anomalies in a child WILLIAMSON *et al* (1963) and KAJII & SHINOHARA (1963) noted haemangiomas in children

POWEL & JOHNSTONE (1962) found serious defects in the diaphragms of two children after the administration of phenmetrazine to the mother WILLIAMSON *et al* (1963) recorded ectopic viscera in chick embryos after thalidomide Abnormalities of the urinary tract have often been noted (PLIESS 1962, WILLIAMSON *et al* 1963) ROBSON & SULLIVAN (1963) found the peritoneal cavity and stomach full of blood in young rabbits after administration of imipramine

Material and Methods

Forty female rabbits of the Danish White Country Breed were used in this experiment All rabbits were from the Danish State Serum Institute They had all been mated previously, 16 of them had had 1 litter each 21 had each had [illegible] and 3 of them [illegible]

Mating

Each doe rabbit was put together with a buck rabbit in a 70 × 70 cm cage for an average of about 5½ hrs A group of 6 or 4 doe rabbits were mated on the same day and the time interval between the mating of groups was never less [illegible]
Vaginal smears were taken at th[illegible]
22 rabbits also during the matin[illegible]
spermatozoa were found in 3 c[illegible]
found in 22 doe rabbits that al[illegible]
any evidence of spermatozoa a[illegible]

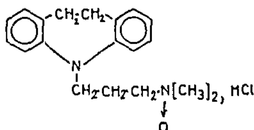


Fig 1 Imipramine-N-oxide hydrochloride

fact may indicate that spermatozoa disappear rapidly from the vagina From 13 of the 22 rabbits showing spermatozoa vaginal smears were made twice in 5 of these spermatozoa were only found once Vaginal smears taken during the long mating period thus seems to be a less safe indicator of a completed mating in rabbits than in rats In this experiment pregnancy occurred more often than would have been expected from the vaginal smears In the seven matings not followed by pregnancy five different buck rabbits were used

Administration of Drugs

All substances were administered daily (including Sundays) on the days indicated in table 1 Subcutaneous injections were given on the assumption that they would result in a more retarded absorption than intraperitoneal and subsequently have a more prolonged action A suspension was made with 10 g thalidomide and 3 75 ml mucilage of acacia, made up to 100 ml with Tyrode's solution One ml/kg corresponding to 100 mg/kg, was administered daily

Imipramine-N-oxide hydrochloride, mol wt 332 86 is shown in fig 1

Two solutions of imipramine-N-oxide HCl were employed 1 5 g substance were dissolved in 30 ml distilled water and 170 ml isotonic pyrogen-free glucose solution two ml of this solution corresponding to 15 mg/kg body-weight the second imipramine-N-oxide HCl solution was made in the same way with 2 5 g of the substance, 50 ml water and 150 ml of the glucose solution, corresponding to 25 mg/kg Imipramine-N-oxide HCl is more soluble in a solution of glucose than in Tyrode's solution The two imipramine hydrochloride solutions were made similarly

The large quantity of 2 ml/kg rabbit was injected to delay absorption and to reduce tissue damage at the site of injection The rabbits in the control group were given 2 ml/kg Tyrode solution daily

The Offspring

Six of the pregnant rabbits were allowed to give birth at term in a normal way 30–32 days after mating The results for these are marked with an asterisk in table 1

The remaining 34 rabbits were anaesthetized with ether on the 30th 31st or 32nd day of gestation The offspring were removed by caesarean section, the membranes were opened, and the umbilical cord was cut after being pinched off The young rabbits were kept warm and their respiration was allowed to begin Foetuses observed moving in the uterus or after the removal were regarded as live and recorded in column 5 in table 1 On going through the reports, however, it could be seen that all foetuses that moved also began breathing After this examination, the dam was killed by opening the thorax

Every one of the young was examined macroscopically for 1) gross appearance,

whether normal or abnormal and 2) the presence, normal development and size of ears, eyes, nose with 2 nostrils, mouth with 4 (6) incisors and tongue, forelegs with 5 toes and hindlegs with 4 toes, and tail and the normal attachment of umbilical cord to the abdominal wall

The findings were recorded, and every abnormal foetus was specially marked with paint and the marking was noted together with the abnormality found After an extrauterine life of ½ hour to 1 hour, the foetuses were killed with ether, marked with clamps, which were placed on different parts of the body and weighed The painted marks, the clamps and the weights ensured an exact record of the origin of each foetus

After opening the abdominal and thoracic cavities, the kidneys were inspected for presence and normal appearance The size and position of certain organs was noted kidneys and adrenals, stomach and intestine (although not stenoses), spleen, diaphragm, liver with gall bladder, urinary bladder, heart and lungs The heart was not opened After the abdominal cavity had been eviscerated, x-radiograms were taken, usually only in a lateral position, though a few were taken of the foetus in a supine position

Almost all films taken in the lateral position were made with the animals placed on their left sides with right forelegs and hindlegs pointing forwards and with left forelegs and hindlegs pointing backward Further, the dorsal side of the right forepaw and the left hindpaw and the plantar side of the left forepaw and the right hindpaw were directed upwards, permitting all the bones of the extremities to be seen separately

Results

A thorough report of the doe rabbits, their offspring and further experimental findings are given in table 1

Key to table 1 List of further anomalies found in the foetuses

a) [illegible]
b) [illegible]
c) [illegible]
d) [illegible] "perforation" of the skull at base [illegible]
e) Open eyes found at the removal of the young one (interpreted as lack of eyelid, eyes are normally found shut)
f) Abnormalities in the abdominal wall at the entrance of the [illegible]
g) [illegible]
h) [illegible]
j) [illegible]
k) [illegible]
l) [illegible]

Table 1

Doe rabbit treated with	No and weight (in kg) of doe rabbit	*Young rabbits born or removed –days after mating*	*Sum of dead foetuses (F) or remains of foetuses (R) in the uterus*	*Sum of live young rabbits born or removed*	Weight (in g) of young rabbits	External and internal appearance of young rabbit *normal*
A						
Thalidomide 100	608 4 00	30	R ?	0		
mg/kg subc ,	625 4 45	30	0	0		
daily from 1 to	559 3 20	30	10 R	0		
17 day after	414 3 55	30	6 R	1	50	
mating				1	51	Yes
				1	51	
B	353 3 35	30	0	0		
	295 3 85		1 F		54	
Thalidomide 100				7	50, 38, 44, 35, 43, 50, 49	Yes
mg/kg subc ,	93 3 50	30	0	0		
daily from 3 to	558 4 50		numerous	1	30	
20 day after			R	1	34	Yes
mating	45 4 20			1	43	
				2	49, 46	
	554 3 65	30	7 R	0		
C	276 4 10	32	10 R		2 rests were not quite small	
				1	54	
				1	63	
Thalidomide 100				1	55	
mg/kg subc ,	660 3 60	31	1 F		50	Yes
daily from 6 to	*			1	44	
20 day after				1	41	
mating				1	35	Yes
				4	49, 49, 47, 50	
	682 3 80	31	0	0		
	601 3 60	31	0	5	35, 26, 45, 39, 40	
				1	36	
D	294 4 20	30	1 R	6	60, 38, 54, 51, 49, 48	Yes
	388 3 20	30	3 R	3	34, 33, 40	Yes
Imipramine HCl	107 4 05	30	0	8	54, 47, 52, 44, 37, 46 38	Yes
25 mg/kg subc ,					54	Yes
daily from 3 to	791 3 65	30	1 R	4	37, 40 36, 46	
20 day after				1	34	
mating	146 3 95	30	0	0		
	545 4 75	32	0	0		
E	693 4 00	30	?	11	26, 30, 39, 26, 22, 30, 29	Yes
Imipramine HCl	*				27, 31 25, 27	
15 mg/kg subc ,	240 3 70	31	?	9	43, 43, 35, 45, 39, 35 40	Yes
daily from 3 to	*				36, 43	
18 day after	795 4 60	31	0	10	42, 39, 24, 31, 26, 25, 29	Yes
mating					27 18, 17	
	337 4 05	31	0	1	69	Yes

Malformed front paws	Malformed front and hind paws	Tail abnormally small or missing	Only 1 kidney present (1) No kidney (0) present	Other abnormalities of various kinds, see key to table 1
	Yes	Yes		see f)
				see f) and g)
Yes				
	Yes	Yes	1	see a) and b)
	Yes	Yes	0	see c) and d)
				see e)
Yes				
		Yes		see h)
				see j)
Yes			1	see h) and k)
?			1	see k)
				Spontaneous death 4 days after [illegible]
			1	[illegible]
?				[illegible]
Weight at age of one month				[illegible]
Yes				
Yes				see l)
			Yes	(normal in other respects)
				Two young rabbits found dead, 9 found alive and were killed all 11 seemed to be [illegible]
Three of the [illegible]				

Doe rabbit treated with	No and weight (in kg) of *doe rabbit*	Young rabbits born or removed – days after mating	Sum of dead foetuses (F) or remains of foetuses (R) in the uterus	Sum of live young rabbits *born* or removed	Weight (in g) of young rabbits	External and internal appearance of young rabbit normal
I						
	782 3 70	30	2 R	11	44, 41, 31, 40, 39, 40, 48 37, 25, 36, 41	Yes
Imipramine-N-oxide HCl 25	642 4 40	30	0	10	35, 53, 53, 53, 46 51, 46 35, 45, 34	Yes
mg/kg subc, daily from 3	419 3 60	30	0	0		
to 18 day	515 4 10	31 1/2	?	5	29, 40, 29, 40, 31	Yes
after mating	•			6	43, 56, 41, 50, 64, 42	Yes
	639 3 90	30	0	0		
	545 4 25	30	1 R	7	28, 39, 35, 28, 42, 35, 29	Yes
G						
Imipramine-N	158 4 25	31 1/2	?	7	60, 47, 46, 57, 40, 60, 45	Yes
-oxide HCl 15	•					
mg/kg subc,	783 4 00	30	1 R	7	45, 36, 35, 33, 32, 36, 41	Yes
daily from3 to	351 4 35	30	1 R	1	53	
18 day after				3	56, 53, 57	Yes
mating	615 4 25	30	1 R	2	49, 51	Yes
H	136 3 55	30	6 R	0		
	554 4 55	32		4	31	Yes
0 9% NaCl	•				53, 63 60	Yes
subc, daily from	385 2 90	30	0	4	61, 55 53 47	Yes
3 to 20 day	460 3 75	30	0	6	55, 56, 53, 52, 50, 54	Yes
after mating	501 3 70	30	0	0		
	767 4 40	30	2 R	10	38, 52, 35, 44, 35, 28, 35 28, 25, 26	Yes

Malformed front paws	Malformed front and hind paws	Tail abnormally small or missing	Only 1 kidney present (1) No kidney (0) present	Other abnormalities of various kinds see key to table 1
Killed with ether after birth Weight at age of one month 405 406 455 470 470 435 g				
Weight at age of one month 390 310 325 430 465 395 390 g				
			1	
Died 2 days after birth At age of one month 680 660 and 660 g				

x-Radiograms

As the examination of the x-radiograms progressed it was found necessary not only to examine the bones of the forelegs, but also to extend radiography to the whole skeleton. The presence and appearance of various bones were subsequently recorded: humerus, radius and ulna seen as two distinctly separate bones, metacarpal bones seen as 4 bones, pelvis seen as 4 bones, separated from each other, femur, tibia and fibula seen as one bone, tarsus seen as one bone, metatarsal bones seen as 4 bones, the vertebral column, consisting of 7 cervical, 12 thoracic, 7 lumbar, and 4 sacral vertebrae, and up to 16 coccygeal vertebrae skull with the normal (number of) teeth in upper and lower jaw, incicors, premolars and molars, and a nasal bone. The size of the fontanelle was measured in mm.

Few of the gross abnormalities that would be obvious also to a non-osteologist were observed. One young rabbit with a protrusion like an elephant trunk on the forehead lacked the upper jaw, including corresponding teeth and nasal bone, but this gross abnormality would have been apparent without x-rays. The 5 young rabbits that had either abnormally small tails or none at all (see table 1, column 10) showed an average of only 5 caudal segments (for further details, see table 2) on x-ray examination, but macroscopic inspection had already shown them to be abnormal on account of the diminutive tail Moreover, the distal segments were so vaguely outlined in many young rabbits with the normal number of tail segments (the same film, the same time of exposure and the same devel-

Table 2

Doe rabbits treated with	Average size of fontanelle in young of the group		Average numbers of countable caudal segments in young of the group	
100 mg thalidomide 1st to 17th day	2 3 mm	2 4 mm	13	7 8
100 mg thalidomide 3rd to 20th day	2 2 mm		7	
100 mg thalidomide 6th to 20th day	2 6 mm		7	
0 9% NaCl 3rd to 20th day	3 8 mm	3 8 mm	11	11 0
15 mg imipramine HCl 3rd to 18th day	3 1 mm	3 2 mm	12	12 2
25 mg imipramine HCl 3rd to 20th day	3 4 mm		13	
15 mg imipramine-N-oxide 3rd to 18th day	2 0 mm	2 2 mm	13	12 3
25 mg imipramine-N-oxide 3rd to 18th day	2 5 mm		12	

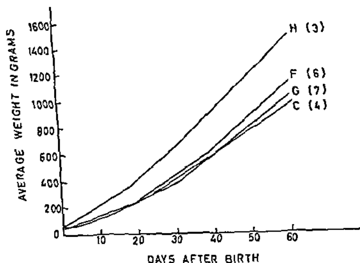


Fig 2 growth of offspring
4 litters from doe rabbits no 660 group C no 554 group H, no 158 group G and no 515 group F See table I

opment technique were used throughout) that the distal segments in another young rabbit with lesser calcification would probably not be seen (counted) even though present Thus an x ray finding of a deficit of caudal vertebrae cannot be in itself a proof of anomaly As a defective development of the bones might result in conspicuously large fontanelles they were measured on all the radiograms Table 2 shows no difference between young from treated and untreated dams

Growth of the Offspring

As can be seen from table 1, there were 6 doe rabbits that gave birth to young One of them [illegible] therefore killed [illegible] of the other fo[illegible] for two months The young from the control group H gave clear evidence of greatest growth, see fig. 2 Whether this was due mainly to the fact that they were the offspring of an untreated rabbit or to the fact that they were *only* three in number, compared with 4–7 on the other three litters, is difficult to say The smallest litter but one, belonging to group C consisted of the young of a thalidomide-treated rabbit, they should have shown a growth curve lying between the curves marked H and F, so there is some possibility of inhibition of growth

If growth depends upon number of young in the litter the curves F and G are consistent As the dams in group F have been given 25 mg,

Table 3

Daily treatment with mg/kg subcutaneously in x–y days after mating	Number of doe rabbits mated and treated	Number of doe rabbits successfully mated, i e pregnancy with dead foetuses or live young	Number of live young in the group	Average number of live young per rabbit in the group	Average number of live calculated *per doe becoming pregnant*	Weight (in grams) of live young per group	Average weight (in grams) per rabbit in the group	Average weight (in grams) of live young calculated per rabbit becoming pregnant	Number of does, producing 1 or more abnormal young/number of does (in the group) in which it was possible macroscopically to see living young	Number of young abnormal in some way (live-born, dead-born or removed)/total number of recordable young
100 mg thalidomide from 1st to 17th day	4	2	3	0.75	1.5	152 g	38 g	76 g	1/1	2/3
100 mg thalidomide from 3rd to 20th day	6	4	12	2.0	3.0	516 g	86 g	129 g	3/3	5/13
100 mg thalidomide from 6th to 20th day	4	3	16	4.0	5.3	708 g	177 g	236 g	3/3	14/19
25 mg imipramine from 3rd to 20th day	6	4	22	3.66 } 5,3	5.50 } 6,62	972 g	162 g } 199	243 g	1/4	1/22
15 mg imipramine from 3rd to 18th day	4	4	31	7.75	7.75	1018 g	254 g	254 g	0/4	0/31
25 mg imipramine-N-oxide from 3rd to 18th day	6	4	39	6.50 } 5,9	9.75 } 7,4	1571 g	262 g } 250	393 g	0/4	0/39
15 mg imipramine-N-oxide from 3rd to 18th day	4	4	20	5.00	5.00	932 g	233 g	233 g	1/4	1/20
0.9% NaCl from 3rd to 20th day	6	5	24	4.0	4.8	1089 g	181 g	218 g	0/4	0/24

but those in group G only 15 mg imipramine N-oxide per day these curves are only consistent if we assume that the drug given to the dam promotes growth of offspring increasing with the dose given This can hardly be assumed At any rate the results do not suggest inhibition of growth

Discussion

According to the results tabulated in tables 1 and 3, the fertility of the rabbits declined more and more the earlier after mating the administration of thalidomide was begun By beginning the administration not before 6 days after mating, not only is the largest number of offspring secured but also the largest number of abnormal young, both in absolute numbers and as a percentage of the total number of young An interval of 6 days seems to be the best Table 3 shows that at an interval of 6 days just as many offspring per rabbit (4) are produced as in the control group receiving saline Apart from the anomalies, the two groups are closely similar in other respects

As the two imipramin drugs would possibly impede pregnancy (ROBSON & SULLIVAN 1963) if administration were begun immediately after mating, we chose to begin administration 3 days after, on the other hand, to begin too late might result in no response, because the proper moment for the occurrence of a teratogenic effect had already been passed For fertility the interval of three days seems to have been well chosen On average more living young per rabbit were found in the two groups receiving imipramine or imipramine-N-oxide than in the control group, when calculated per pregnant rabbit Further, the imipramine treated rabbits gave birth to a greater weight of living young, both on average per rabbit and on average per rabbit that became pregnant, than did the control rabbits As for dead foetuses or partly resorbed foetuses, table 4

Table 4

Treatment with	Number of rabbits with dead foetuses or remains of foetuses*/ number of rabbits becoming pregnant	Number of dead foetuses or remains of foetuses
Thalidomide	7 (8)/9	ca 40
0 9% NaCl	2/5	8
Imipramine HCl	3/8	5
Imipramine N-oxide HCl	5/8	6

* Besides living young ones in most cases

shows that many of the thalidomide treated rabbits contained so many of them that these rabbits appear different from those in the 5 (3) other groups, though these can hardly be said to differ from one another

As for the teratogenic effect, tables 1 and 3 demonstrate that all thalidomide treated rabbits in which the foetuses were big enough to permit reliable inspection showed at least one abnormal young per litter The most frequent anomalies can be seen from table 1, and other anomalies are recorded in the key to table 1 It should be remarked here that whether "malformed front and hind paws" are to be considered a teratogenic or an embryotoxic effect is not clear, because we have not observed whether young rabbits with malformed paws, if left alive, develop towards normal and we have not conducted histological examinations to decide whether or not the tissue in the paws consisted of normal cells in a normal state

From table 3 it can be seen that 21 out of 35 young were abnormal in some way Of the imipramine-treated rabbits' young, one out of 53 was found abnormal with an extremely short tail, among the imipramine N-oxide treated rabbits' young, one out of 59 was found abnormal in having only one kidney These abnormalities were not found in the control group, but so often in the thalidomide groups that here at least the possibility of co incidence must be rejected It will be reasonable therefore, to accept that the effect of imipramine or imipramine N oxide has been the cause of the abnormality of the two young

As for the x ray examinations, one might perhaps consider them superfluous or even misleading But the picture of a skeleton, where the only abnormal thing seems to be that the metacarpal and metatarsal bones or in some instances the whole skeleton are reproduced faintly and that some caudal segments are absent, is a result of some value It is beforehand impossible to predict with certainty whether or not a drug will in particular produce anomalies in the skeleton

Summary

Forty female rabbits of the white Danish breed were mated, and 14 were given thalidomide daily for a fixed period, 10 received imipramine HCl, 10 received imipramine-N-oxide and 6 controls received saline solution All injections were subcutaneous

Macroscopic examination showed that 22 of 35 young of thalidomide treated rabbits were abnormal in some way, one of 53 young of imipramine treated rabbits and one of 59 young of imipramine-N oxide treated rabbits were abnormal, whereas no young from the control group of 25 were abnormal The effect of thalidomide depended on the time between

mating and beginning administration Thalidomide in a dose of 100 mg/kg markedly reduced the fertility, but imipramine HCl and imipramine-N oxide (15 mg or 25 mg/kg daily) did not Thus, the white Danish rabbit can be used for investigating the teratogenic effects of thalidomide-like substances

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REFERENCES

Ciba foundation symposium *Congenital malformations*, London 1960

Christie G A Thalidomide and congenital abnormalities *Lancet* 1962, II, 249

Ehmann, B Teratogenic effects of thalidomide *Lancet* 1963, I, 772

Felisati, D Thalidomide and congenital abnormalities *Lancet* 1962, II, 724–725

Giroud A, H Tuchman Duplessis & L Mercier Parot Thalidomide and congenital abnormalities *Lancet* 1962, *II* 298–299

Grainger, R G, A H Morris & P Ward Phocomelic deformity and maternal thalidomide administration report of four cases *Brit J Radiol* 1962, 35, 687–691

Hagen, Odette Drugs and congenital abnormalities *Lancet* 1963, I, 501

Kajii, Tadashi & Mamoru Shinohara Thalidomide in Japan. *Lancet* 1963, I, 501–502

Kalter, H & J Warkany Experimental production of congenital malformations in mammals by metabolic procedure *Physiol Rev* 1959 *39* 69–115

Kemper, F Thalidomid und entwicklung von Hühner-embryonen *Arzneimittel forschung* 1962, 12, 640–641

Leck, I M & E. L. M Millar Incidence of malformations since the [illegible]

[illegible] and congenital abnormalities *Lancet* 1961, II, 1358

McColl, J D, M. Globus & S Rob[illegible] tal mal[illegible] *Engl J* [illegible] ies. *Brit*

Murphy M [illegible]

Pliess [illegible] abnormalities *Lancet* 1962, I, 1128–1129

Powell, P D & J M Johnstone Phenmetrazine and foetal abnormalities *Brit Med J* 1962, (5315) 1327

Robson, J M [illegible]

Rus [illegible]

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From the Research Division of Pharmacia Ltd,
Copenhagen, Vanløse, Denmark

Some Pharmacological Properties of a New Adrenergic Neurone-blocking Agent. N-(β-guanidinoethyl)-hexahydrobenzo-[d]-azocine sulphate (Ph 881/7)

By

Keld Hermansen

(Received July 20 1963)

The first compound in the field of adrenergic neurone-blocking agents was *TM 10* (Fig 1a) [illegible]

[illegible] that TM 10 interferes with the last stage in the biosynthesis of noradrenaline by inhibiting dopamine dehydrogenase (BAIN & FIELDEN 1957) A preliminary trial on patients with high blood pressure indicated that it was effective as a hypotensive agent, but too toxic for clinical use

Another compound in the same field is *bretylium* (*Fig 1b*), which appeared in 1959 (BOURA & GREEN) From its extensive use in the treatment of hypertension it is known to possess certain disadvantages, such as poor absorption from the gastro intestinal tract due to its quaternary

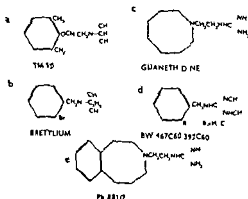


Fig. 1 The chemical structure of some adrenergic neurone-blocking compounds.

Seller, Mary J Thalidomide and congenital abnormalities *Lancet* 1962 II, 249

Smithells, R W Thalidomide and congenital abnormalities *Lancet* 1962 *I, 591*

Somers, G F Thalidomide and congenital abnormalities *Lancet* 1962 I 912-913

Speirs, A L. Thalidomide and congenital abnormalities *Lancet* 1962 I 303-305

Wiedemann, *Hans* Rudolf Hinweis auf derzeitige häufung hypo und aplastischer fehlbildungen der gliedmassen *Med Welt* 1961, pp 1863-1866

Williamson, A P, R J Blattner & H R Lutz Abnormalities in chick embryos following thalidomide and other insoluble compounds in the amniotic cavity *Proc soc exp biol 1963, 112, 1022-1025*

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The first compound in the field of adrenergic neurone-blocking agents was TM 10 (Fig 1a) introduced by HEY & WILLEY in 1954 These authors related the blocking action to its powerful local anaesthetic activity, an interpretation that was questioned by EXLEY (1957) Later it became evident that TM 10 interferes with the last stage in the biosynthesis of noradrenaline by inhibiting dopamine dehydrogenase (BAIN & FIELDEN 1957) A preliminary trial on patients with high blood pressure indicated that it was effective as a hypotensive agent, but too toxic for clinical use

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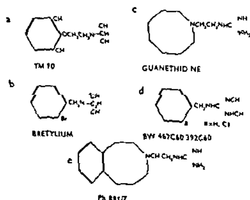


Fig. 1 The chemical structure of some adrenergic neurone blocking compounds

structure, a high frequency of side effects (TURNER 1960) and development of tolerance in man

The first observations on guanethidine (Fig 1c) were published in 1960 by MAXWELL et al In the last two years this compound has received intensive pharmacological study, and in the clinic it has proved to be of value in the management of hypertension

Recently a new series of adrenergic neurone blockers has been reported by BOURA et al (1961) They (Fig 1d) are derivatives of bretylium in which the quaternary part is replaced by a dimethylated guanidino group the adrenergic blocking activity is claimed to be twice that of guanethidine

This paper deals with the pharmacological properties of Ph 881/7*) (Fig 1e), which was synthesized by HJELTE et al in the Research Division of AB Pharmacia, Uppsala, Sweden It is distinct from guanethidine in having a benzene ring fused to the 8-membered azocine ring In animal experiments Ph 881/7 shows properties remarkably different from those of guanethidine

Methods and Results

The compounds used were N (β guanidinoethyl) hexahydrobenzo [d] azocine sulphate (Ph 881/7) containing 82 1% of the base, guanethidine sulphate containing 80 1% of the base adrenaline (adrenaline MCO) noradrenaline bitartrate amphetamine sulphate, D amphetamine sulphate and hexamethonium bromide (vegolysen ® M & B) All doses recorded below refer to the salts, except those of adrenaline and noradrenaline, for which the dose of free base is given

Effect of Ph 881/7 in the unanaesthetized Animal

Mouse Only slight symptoms were seen at subtoxic dose levels (<50 mg i p) These consisted of transient mydriasis, diarrhoea and muscle weakness The mice, however, were not curarized even by sublethal doses being able to keep their positions on an inclined screen

Rat Rats were given continous intravenous injections of Ph 881/7 through a polythene catheter previously placed in the jugular vein As small a dose as 1 mg/kg i v caused some signs of central stimulation the animals becoming aroused and beginning to scratch their noses Further, they became hyperactive and tried to escape from their cages When the intravenous injection was continued, the rats became depressed by a dose of 10 mg/kg Next, grinding the teeth and momentary seizures often appeared, if the speed of injection did not exceed 1 mg/kg/min , approx

*) A preliminary communication on this compound was given at the Scandinavian Summer Meeting of Pharmacology at Göteborg 1962

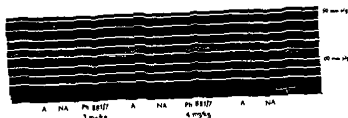


Fig 2 The effect of Ph 881/7 on blood pressure of the unanaesthetized rat A = 0 5 µg/kg adrenaline NA = 0 5 µg/kg noradrenaline For details see text

30 mg/kg could be given before death occured A simultaneous blood pressure recording (Fig 2) was made of these rats through a catheter inserted in the common carotid artery It will be noted that 1 mg/kg as well as 4 mg/kg of Ph 881/7 i v caused slight transient hypertension After 4 mg/kg the pressor response to adrenaline and noradrenaline was potentiated

Cat In the cat 20 mg/kg s c caused maximal relaxation of the nictitating membrane after a latency period of 20 min This effect persisted for 2 days At the same time moderate bradycardia and diarrhoea appeared, these symptoms were only transient, whereas the tone of the nictitating membrane did not return to normal until 8 days later For comparison the same dose of guanethidine was given The onset of action of this substance was much slower than that of Ph 881/7; the maximal relaxation did not occur until about 7 hr after the subcutaneous injection The tone of the membrane was normal after 10 days

Haemodynamic action of Ph 881/7 in anaesthetized normotensive cats

The effect of Ph 881/7 on blood pressure in anaesthetized animals has been tested on cats anaesthetized with chloralose (100 mg/kg i p) or allypropymal (75 mg/kg i p) Both were used because preliminary experiments had shown that the initial hypertensive phase after bretylium and guanethidine was less pronounced in allypropymal than in chloralose anaesthesia This finding is in agreement with those of EMMELIN & STRÖMBLAD (1954) who observed that chloralose does not deplete the adrenaline and noradrenaline stores of the adrenals as do some other anaesthetics This probably means that more is available for different amine releasing compounds introduced during the chloralose anaesthesia As the adrenergic neurone blocking agents possess a sympathomimetic pressor component one might *therefore speculate* that this component may conceal a possible hypotensive effect of the compound tested in an acute experiment. This would not be so when allypropymal is used because the initial hypertensive phase of amine-releasing compounds is less pronounced in this anaesthesia

Fig 3a shows the effect of 5 mg/kg of Ph 881/7 i v on a chloralose anaesthetized cat Apart from a transient hypertension, there was an

structure, a high frequency of side effects (TURNER 1960) and development of tolerance in man

The first observations on guanethidine (Fig 1c) were published in 1960 by MAXWELL *et al* In the last two years this compound has received intensive pharmacological study, and in the clinic it has proved to be of value *in the management of hypertension*

Recently a new series of adrenergic neurone blockers has been reported by BOURA *et al* (1961) They (Fig 1d) are derivatives of bretylium in which the quaternary part is replaced by a dimethylated guanidino group the adrenergic blocking activity is claimed to be twice that of guanethidine

This paper deals with the pharmacological properties of Ph 881/7*) (Fig 1e), which was synthesized by HJELTE *et al* in the Research Division of AB Pharmacia, Uppsala, Sweden It is distinct from guanethidine in having a benzene ring fused to the 8 membered azocine ring In animal experiments Ph 881/7 shows properties remarkably different from those of guanethidine

Methods and Results

The compounds used were N (β guanidinoethyl) hexahydrobenzo [d] azocine sulphate (Ph 881/7) containing 82 1 % of the base guanethidine sulphate containing 80 1 % of the base, adrenaline (adrenaline MCO) noradrenaline bitartrate amphetamine sulphate, D amphetamine sulphate and hexamethonium bromide (vegolysen ® M & B) All doses recorded below refer to the salts, except those of adrenaline and noradrenaline, for which the dose of free base is given

Effect of Ph 881/7 in the unanaesthetized Animal

Mouse Only slight symptoms were seen at subtoxic dose levels (<50 mg i p) These consisted of transient mydriasis, diarrhoea and muscle weakness The mice, however, were not curarized even by sublethal doses, being able to keep their positions on an inclined screen

Rat Rats were given continous intravenous injections of Ph 881/7 through a polythene catheter previously placed in the jugular vein As small a dose as 1 mg/kg i v caused some signs of central stimulation the animals becoming aroused and beginning to scratch their noses Further, they became hyperactive and tried to escape from their cages When the intravenous injection was continued, the rats became depressed by a dose of 10 mg/kg Next, grinding *the teeth and momentary seizures* often appeared, if the speed of injection did not exceed 1 mg/kg/min , approx

*) A preliminary communication on this compound was given at the Scandinavian Summer Meeting of Pharmacology at Göteborg 1962

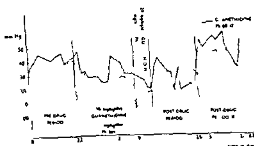


Fig 4 Hypotensive effect of guanethidine or Ph 881/7 mixed in the feed of renal hypertensive rats For description see text

After a pre treatment period of 2 weeks, sufficient Ph 881/7 or guanethidine was mixed in the feed to give a dose of approx 10 mg/kg/day Later the concentration in the feed was increased 3 times After the end of the medication, blood pressure determinations were continued until the pressure returned to the initial level

In Fig 4 the results from such an experiment as that described above are plotted graphically The average blood pressure in the pre treatment period was 140 mm Hg for both groups At 10 mg/kg/day it decreased by about 15 mm, at 30 mg/kg/day the average was 30 mm below the initial level When the rats were fed on a normal diet again, the blood pressure increased gradually and reached the pre treatment level after 2 weeks The hypertensive effect of Ph 881/7 was moderate, probably because the rats were only slightly hypertensive initially From this experiment one might still conclude that Ph 881/7 has a hypotensive effect when given by mouth and that its potency is of the same order as that of guanethidine

Effect of Ph 881/7 on autonomic nerve transmission

For these experiments cats were anaesthetized with 0 1 g/kg chloralose i p The compounds to be tested were injected into the saphenous vein As indication of unaffected sympathetic pathway the maximal response was chosen of the nictitating membrane to an electrical stimulation of the cervical sympathetic nerve The nerve was stimulated either pre ganglionically or post ganglionically by square wave impulses at a frequency of 15 sec, the pulse length being 15 msec and the voltage 1 5–6 0 V

As an indication of unaffected parasympathetic nerve transmission the maximal fall was chosen in blood pressure elicited by electrical stimulation of the peripheral end of the cut vagus nerve in the neck the stimulation parameters being as described above The blood pressure was recorded in the right carotid artery

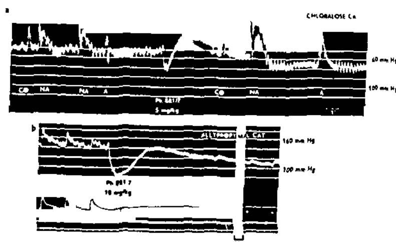


Fig 3 Blood pressure analyses of Ph 881/7 and different anaesthetics

a) Blood pressure tracing from a chloralose anaesthetized cat (100 mg/kg i p)
CO = carotid occlusion for 15 sec, NA = 0 5 µg/kg noradrenaline, A = 2 5 µg/kg adrenaline

b) Allypropymalanaesthetized cat (75 mg/kg i p)
The upper tracing is the blood pressure curve The second tracing shows contractions of the nictitating membrane in response to electrical stimulation of the cervical sympathetic trunk at the dots
The third tracing is a record of the respiration The bottom record indicates the time in min

For further description see text.

insignificant decrease in blood pressure (20 mm Hg) The carotid occlusion reflex (CO) was inhibited, but the noradrenaline (NA) and adrenaline (A) responses were enhanced *It is noted that the hypertensive phase of* Ph 881/7 is small, especially compared with that observed after guanethidine

Fig 3b illustrates an experiment with an allypropymal anaesthetized cat, to which 10 mg/kg of Ph 881/7 were given i v In spite of the higher dose, the hypertensive phase was smaller than in the experiment recorded in Fig 3a Further, it is seen that this dose caused a significant fall in blood pressure (40 mm Hg), which was, however, not always reproducible

It may therefore be said that Ph 881/7 has no significant effect on blood pressure in normotensive cats or rats (*Fig 2*)

Hypotensive effect in renal hypertensive rats

Male rats weighing 200–300 gms were used Hypertension was induced by clamping the renal arteries with silver clips, as described by Wilson & Byron (1939) Two months after the operation, *moderate hypertension developed, when the blood pressure had been constant for a week, two groups of 6 rats were selected* Blood pressure determinations were carried out as described by Bing (1956)

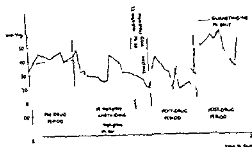


Fig 4 Hypotensive effect of guanethidine or Ph 881/7 mixed in the feed of renal hypertensive rats For description see text

After a pre treatment period of 2 weeks sufficient Ph 881/7 or guanethidine was mixed in the feed to give a dose of approx 10 mg/kg/day Later the concentration in the feed was increased 3 times After the end of the medication, blood pressure determinations were continued until the pressure returned to the initial level

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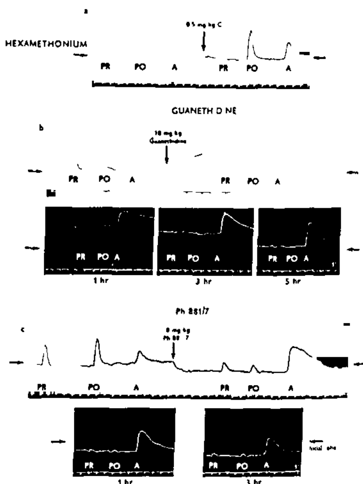


Fig 5 The effect of hexamethonium (C_6), guanethidine or Ph 881/7 on ganglionic transmission

The figure depicts contractions of the nictitating membrane in the cat The compounds were applied where indicated by [illegible] electrical stimulation of the ce [illegible]
Fig 5a shows that 0 5 mg/kg [illegible] augmented [illegible] responses and left the membrane hypersensitive to adrenaline

Fig 5 demonstrates the effect of hexamethonium, guanethidine and Ph 881/7 on transmission across the superior cervical ganglion in the cat Contractions of the nictitating membrane were elicited either by electrical stimulation of the cervical sympathetic trunk or by intravenous injection of adrenaline As seen from Fig 5, Ph 881/7 blocked pre- and postganglionic nerve stimulation equally well, as did guanethidine, without decreasing the sensitivity of the nictitating membrane to injected adrena-

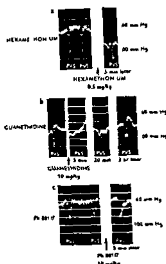


Fig 6 The effect of hexamethonium (C_6), guanethidine or Ph 881/7 on the depressor response caused by electrical stimulation of the cut vagus nerve to the heart (PVS) in the cat

Fig 6a shows that C_6 lowered the blood pressure and caused a partial block of the PVS response

Fig 6b shows that guanethidine blocked the peripheral vagal stimulation initially, but that it was restored 3 hr later

Fig 6c shows that Ph 881/7 left the PVS response unaffected

line This was in fact augmented in presence of either substances The surprising difference between guanethidine and Ph 881/7 was that the latter caused a small relaxation, whereas the former caused a long lasting (>5 hr) and marked contraction of the membrane

Fig 6 shows the effects of hexamethonium, guanethidine and Ph 881/7 on the fall in blood pressure initiated by electrical stimulation of the peripheral end of the cut vagus nerve in the neck It shows that hexamethonium inhibited the response to stimulation of the peripheral vagus (PVS) whereas guanethidine caused immediately after the injection complete abolition of this response, which was not restored until 5 hr later The bottom tracing shows that 10 mg/kg of Ph 881/7 i v – a dose that always blocked sympathetic nerve transmission – left the PVS response unaffected In other experiments there was possibly a transient block of the PVS response also after Ph 881/7

From the experiments illustrated in Fig 5 and Fig 6 it must be concluded that Ph 881/7 blocks sympathetic nerve transmission at a site peripheral to the ganglion and leaves the nictitating membrane hypersensitive to injected adrenaline Further, Ph 881/7 has no direct or indirect

effect upon the membrane as is found with guanethidine, which always causes a long lasting contraction

Anti-amphetamine effect of Ph 881/7 on blood pressure in the rat

MAXWELL *et al* (1960) demonstrated a clear anti amphetamine effect of guanethidine in the pentobarbital anaesthetized dog when the substance was given 48 hr before the experiment In order to see if Ph 881/7 showed the same effect the technique was adapted to rats They were anaesthetized with urethane (1 5 g/kg s c.) I v injections were made via a polythene catheter in the right jugular vein the blood pressure being recorded manometrically via a glass cannula in the left carotid artery

The dose of amphetamine sulphate was 1 mg/kg i v The rats showed pronounced development of tachyphylaxis towards amphetamine Consequently reproducible increases in blood pressure from subsequent injections could not be obtained, and it was found necessary to use a control group of untreated rats Each rat was thus only injected once with amphetamine, and the responses of the untreated ones were compared with those of the rats that had received guanethidine or Ph 881/7 24 hr or 48 hr before amphetamine The results obtained are given in table 1

As seen from table 1, guanethidine showed no anti amphetamine effect at all, either after 24 hr or 48 hr Ph 881/7 reduced the response when it was given 48 hr before amphetamine It was indeed surprising that the results on the dog were not reproducible in the rat, because it is known from the work with other compounds in the same series that this animal is well able to develop a complete resistance to amphetamine

Table 1

Anti amphetamine effect of Ph 881/7 and guanethidine in rats

Compound	No of exp	Given s c hr before amphetamine	Amphetamine response mm Hg	Amphetamine response %	Result*)
Control	11		13 ± 3**)	16 ± 4**)	
Guanethidine	2	24	14	14	0
	2	48	15	22	0
Ph 881/7	4	24	14	18	0
	4	48	9	10	(+)

*) 0 indicates that the amphetamine response was unaffected
(+) indicates that the amphetamine response was reduced
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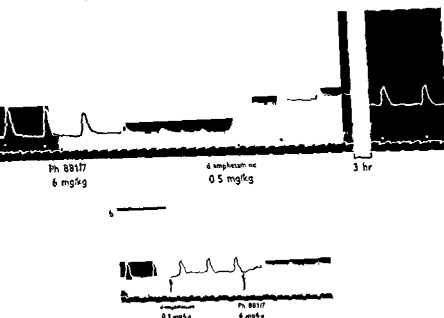


Fig 7 The antagonizing effect of d amphetamine on *Ph 881/7* induced block
The tracings show contractions of the *nictitating* membrane in the cat. The dots indicate the time at which the cervical sympathetic nerve was electrically stimulated
For details see text

Antagonism between D *amphetamine and Ph 881/7 in the cat*

As shown by DAY (1962) it is possible to overcome a guanethidine induced block of the sympathetic nerves by giving D amphetamine sulphate He also showed that the blocking effect of guanethidine did not appear if D *amphetamine* was given first

Similar experiments were carried out with *Ph 881/7*, as shown in Fig 7 Fig 7a shows the contractions of the nictitating membrane being blocked by 6 mg/kg of Ph 881/7 When 0 5 mg/kg of D-amphetamine was then given it caused an almost complete recovery after 3 hr On the other hand, when 0 1 mg/kg of D amphetamine was given before 6 mg/kg of Ph 881/7, it was unable to antagonize the blocking effect of Ph 881/7, as shown in Fig 7b

Toxicology

Acute Toxicity

LD100 was determined by intravenous injection in mice by the method of RICHTER (1958) the speed of injection being approximately 5 mg/kg/

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Control	11		13 ± 3**)	16 ± 4**)	
Guanethidine	2 2	24 48	14 15	14 22	0 0
Ph 881/7	4 4	24 48	14 9	18 10	0 (+)

*) 0 indicates that the amphetamine response was unaffected
(+) indicates that the amphetamine response was reduced
**) s

luced by guanethidine If mice were pre treated with the monoamine oxidase inhibitor iproniazid and if 24 hr later guanethidine was given, they showed the characteristic picture of noradrenaline release hyperactivity, piloerection and mydriasis This was not so when Ph 881/7 was administered Whether this difference may prove to be an advantage in the treatment of hypertensive patients is still in question

The strong sympathomimetic properties of guanethidine may account for the delayed relaxation of the nictitating membrane in unanaesthetized cats when compared with that of Ph 881/7 This difference in onset of action also may have clinical consequences

Summary

In anaesthetized cats 10 mg/kg i v of Ph 881/7, a new guanidine derivative, blocks sympathetic nerve transmission at some site between the superior cervical ganglion and the nictitating membrane without antagonizing the effects of adrenaline and noradrenaline It lacks the initial sympathomimetic action of guanethidine, which suggests a weaker amine releasing capacity or none Peripheral vagus stimulation is unaffected or only transiently depressed Ph 881/7 has little or no influence on the blood pressure of normotensive animals, but it suppresses that of the renal hypertensive rat

Acknowledgements

The author acknowledges the valuable assistance of Mrs B Petersen and of Miss A Schytt

REFERENCES

Ba n W A & R Fielden *In vitro* formation of noradrenaline from dopa m ne by human tissue *Lancet* 1957 *II* 472–473

B ng I Effects of ether anaesthesia on the results of indirect determination of blood pressure in the rat *Acta pharmacol et toxicol* 1956 12 285–293

Boura A L. A F C Copp A F Green H F Hodson G K. Ruffell M F Sim E Walton & E. M Grivsky Adrenergic neurone-blocking agents related to choline 2 6-xylyl-ether bromide (TM 10) bretyl um and guanethid ne *Nature* 1961 191 1312 1313

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guanethid ne br
421–439

Emmel n N & R Strömblad Adrenaline and noradrenaline content of the suprarenals of cats in chloralose and morphine-ether anaesthesia *Acta Physiol Scand* 1952, 24 261 266.

Exley K A The block ng action of choline 2 6 xylyl ether bromide on adrenergic nerves. *Brit J Pharmacol* 1957 12, 297–305

min LD50 (24 hr) was determined after intraperitoneal or oral administration Ten mice were used at each dose level and the results were calculated by the method of LITCHFIELD & WILCOXON (1949) The results obtained were

LD100 mice i v 14 5 ± 3 2 mg/kg (n = 10)
LD50 mice i p 83 (76–91) mg/kg (n = 40)
LD50 mice p o 1225 (980–1530) mg/kg (n = 40)

Sub-chronic Toxicity

Sub chronic toxicity of Ph 881/7 was investigated on male rats No macroscopic or histopathological changes of thyroid glands, lungs, heart liver, spleen, kidneys, adrenals, testes or bone marrow to be ascribed to drug administration were observed after oral administration of 9 6, 28 or 84 mg/kg/day for 124 days, nor were any changes in the blood picture caused by these doses No changes in behaviour or in increase in weight occurred during the test period

Discussion

The toxicological results for the LD50 i p and LD50 p o indicate that the absorption of Ph 881/7 from the gastro intestinal tract is only moderate or slow Experiments with hypertensive rats, however, showed that oral Ph 881/7 also exerts a hypotensive effect equal to that of guanethidine

One striking difference between guanethidine and Ph 881/7 revealed by the experimental results is in the latter's lack of sympathomimetic properties It seems to be a qualitative one, as the contracting effect of guanethidine on the nictitating membrane (Fig 5) was very marked and long lasting (>5 hr) whereas Ph 881/7 entirely lacked this action in spite of the fact that the doses used were equipotent in adrenergic neurone blocking effect Guanethidine also caused a sustained blood pressure rise (>2 hr) in the cat, whereas that of Ph 881/7 was only transient (Fig 3)

It now seems well established that the initial sympathomimetic phase of guanethidine is due to release of noradrenaline from endogenous stores This action, however, may well be unrelated to the blocking properties, as the sympathetic block of guanethidine is fully developed before the tissue is depleted of noradrenaline (MCCUBBIN *et al* 1961) Therefore a possible weaker amine releasing capacity of Ph 881/7 does not necessarily indicate a weaker blocking effect

Another type of experiment suggested by RICHTER (1962) showed that the release of amines after Ph 881/7 was small compared with that pro-

cta pharmacol et toxicol 1963, 20, 213–221

From the Department of Pharmacology, University of Copenhagen,
(Professor Knud O Møller, M D)

Analgesic Activity and Brain Concentration of Morphine in Tolerant and Non-Tolerant Rats given Morphine alone or with Neostigmine

By

Torkell Jóhannesson and Jens Schou

(Received July 25, 1963)

Numerous studies have been performed in attempts to elucidate the mechanism of tolerance development against the analgesic and other pharmacological effects of morphine Several possibilities could explain development of tolerance, such as decreased sensitivity of the receptors in the central nervous system or an altered fate of morphine in the tolerant organisms that could lead to a decrease in concentration at the site of action The latter possibility would imply an altered distribution pattern of the drug caused, for example, by a diminished permeability of the blood brain barrier or a lower concentration in the blood of the freely diffusible form of morphine This might be produced not only by increased binding of the drug to plasma proteins and glucuronide formation, but also by a delayed absorption rate or an increased rate of excretion The validity of the hypothesis of an altered biological fate of morphine in the tolerant organism has been tried in experiments on tolerant and non tolerant rats or dogs (WOODS 1954, SZERB & MCCURDY 1956, JÓHANNESSON (1962a & b) Recently MULE & WOODS (1962) used N-^{14}C-methyl labeled morphine to study the distribution of the substance in the central nervous system of tolerant and non tolerant dogs after subcutaneous administration of 2 mg/kg However, none of these authors attempted a comparison of the analgesic effect and the concentration in the brain of morphine after low analgesic doses given to both tolerant and non-tolerant rats

SLAUGHTER and associates reported in 1940 (a & b) that neostigmine increased the analgesic effect of morphine in cats and human subjects Since then many authors have reported that several cholinergic agents,

Hey, P & G L Willey Choline 2 6 xylyl ether bromide an active quaternary local anaesthetic *Brit J Pharmacol* 1954, 9, 471–475

Litchfield, J T & F Wilcoxon A simplified method of evaluating dose effect experiments *J Pharmacol* 1949, 96, 99–113

Maxwell, R A, A J Plummer, F Schneider, H Povalski & A I Daniel Pharmacology of [2 (octahydro-1 azocinyl) ethyl] -guanidine sulfate (SU-5864) *J Pharmacol exp Therap* 1960, 128, 22–29

McCubbin, J W, Y Kaneko & I H Page The peripheral cardiovascular actions of guanethidine in dogs *J Pharmacol exp Therap* 1961, 131, 346–354

Richter, A W Estimation of the i v mean lethal dose for mice by a constant infusion titration *Acta pharmacol et toxicol* 1958, 15, 37–42

Richter, A W Personal communication 1962

[illegible]

1939, I, 136–139

intraperitoneally in amounts of 1 0 mg/kg 3½ hours before measuring the degree of analgesia The rats on this dosage were affected by diarrhoea

The analgesic effect of morphine was determined by giving successive electrical stimuli each lasting 20 millisec , of defined voltage (3 volts) and frequency (1 Hertz) through electrodes inserted into the tails of the animals The technique has been described in detail by JÓHANNESSON & SCHOU (1963) In the previous investigation 3 doses of each of the analgesics employed (morphine codeine and normorphine) were used and apparent rectilinearity and parallelism was obtained between the measured analgesia and the given doses, both plotted as logarithms In the investigation reported here we therefore reduced the number of dosage levels to two for each experimental condition though aware that this might lower the significance of there sults The normal pain thresholds of the animals were established immediately before administration of the morphine Morphine was then injected, and the analgesic effect was determined 30 minutes later Neostigmine or reserpine, when given alone, had no analgesic effect in our animals

Experiments on degree of analgesia The analgesic effects of 5 mg/kg and 10 mg/kg of morphine were determined in non tolerant rats To be able to evaluate any possible influence of neostigmine on morphine analgesia, morphine plus neostigmine were given to another group of non tolerant rats, and the analgesic effect was determined.

The morphine tolerant rats were put on to experiment 17–20 hours after the last daily dose of morph ne At th

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The ... of reserpine on morphine analgesia was investigated in acute experiments Non tolerant rats were given reserpine by intraperitoneal injections 3½ hours later they were given 10 mg/kg of morphine The analgesic effect was determined 30 minutes later

To be able to evaluate any possible effect of neostigmine on the concentration of morphine in the brain of morphine tolerant and non tolerant rats, a representative number of rats that had received morphine (10 mg/kg) alone or morphine plus neostigmine were killed immediately after the analgesic effect had been determined Their brains (including the brain stem and the cerebellum) were taken out and homogenized Morphine was determined either in brain homogenates from single animals or in pooled homogenates of brains from two animals

Determinations of morphine were performed polarograph

... the last injection of morphine, was taken though the analytical procedure Its polarogram gave no reading (*n d*) This symbol is used whenever no morphine was found

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... quantitatively evaluated, is entered as 0 10 µg/g (cf JOHANNESSON & SCHOU 1963) The symbol *n d* was entered as 0 A χ^2-test was performed, as described by BURN (1950) where mentioned in presenting the results of measuring the degree of analgesia

for example neostigmine and physostigmine, can potentiate the effect of centrally acting analgesics such as morphine and pethidine (e g FLODMARK & WRAMMER 1945, CHRISTENSEN & GROSS 1948, KOMLOS *et al* 1950, SLAUGHTER 1950, PÓRSZÁSZ *et al* 1951, SZERB 1957, SAXENA 1958 SCHAUMANN 1959) However, in contrast with these findings ANDREWS (1942), DE JONGH (1954) and HERKEN, MAIBAUER & MULLER (1957) could not confirm that neostigmine increases the analgesic action of morphine It is also controversial whether or not neostigmine administration leads to a higher brain concentration of morphine after a given dosage of this drug, as was suggested by KNOLL, KOMLÓS & TARDOS (1953) SZERB & McCURDY (1956) injected neostigmine subcutaneously 20 minutes before intravenous injection of morphine (30 mg/kg) and found no effect on the brain concentration of morphine Further, JÓHANNESSON (1962b) found no significant difference between the concentrations of morphine in the brain of rats given high toxic amounts of morphine intraperitoneally with or without neostigmine

The intention of the experiments reported below was to measure in parallel the extent of analgesia and the brain concentrations of morphine in tolerant and non-tolerant rats Further, we duplicated these experiments with the addition of injections of neostigmine at the same time as the administration of morphine in an attempt to solve the problems mentioned above Finally, rats of a single group were pretreated with reserpine before measuring the degree of analgesia to test the assertion of SCHAUMANN (1958) that morphine analgesia is mediated by release of catecholamines

Methods

Adult male albino rats (130–220 g) were used Morphine tolerance in the rats was produced as previously described (JÓHANNESSON 1962a) The normal rats are referred to as non tolerant rats

Doses and injection solutions Morphine chloride was dissolved in saline (0 9%) and injected intraperitoneally by means of a tuberculin syringe calibrated to deliver 0 01 ml Morphine was given in amounts of 5 mg/kg 10 mg/kg or 20 mg/kg The concentrations were adjusted so that the injected volumes always amounted to 2 ml/kg

Neostigmine bromide was also dissolved in saline (0 9%) The injection solution contained 0 1 mg/ml and the dose was 0 1 mg/kg This dose gave no fasciculations in our animals When giving both morphine and neostigmine to the same animal we injected morphine first and then neostigmine from another syringe immediately afterwards

Reserpine (Serpasil ®) 2 5 mg/ml) was diluted with saline to contain 0 5 mg ml The injection solution was prepared immediately before use Injections were given

intraperitoneally in amounts of 1 0 mg/kg 3½ hours before measuring the degree of analgesia The rats on this dosage were affected by diarrhoea

The analgesic effect of morphine was determined by giving successive electrical stimuli, each lasting 20 millisec, of defined voltage (3 volts) and frequency (1 Hertz) through electrodes inserted into the tails of the animals The technique has been described in detail by JÓHANNESSON & SCHOU (1963) In the previous investigation 3 doses of each of the analgesics employed (morphine, codeine and normorphine) were used, and apparent rectilinearity and parallelism was obtained between the measured analgesia and the given doses, both plotted as logarithms In the investigation reported here we therefore reduced the number of dosage levels to two for each experimental condition, though aware that this might lower the significance of there sults The normal pain thresholds of the animals were established immediately before administration of the morphine Morphine was then injected, and the analgesic effect was determined 30 minutes later Neostigmine or reserpine, when given alone, had no analgesic effect in our animals

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The morphine tolerant rats were put on to experiment 17–20 hours after the last daily dose of morphine At this time their pain threshold did not differ from that of nontolerant controls, and no demonstrable amounts of morphine could be measured in the brain by the analytical procedure used (see below) Lower doses than 10 mg/kg of morphine had no measurable analgesic effect on the tolerant rats They were therefore given 10 mg/kg and 20 mg/kg of morphine For comparison, the analgesic effect was determined in morphine tolerant rats receiving morphine plus neostigmine

The influence of reserpine on morphine analgesia was investigated in acute experiments Non-tolerant rats were given reserpine by intraperitoneal injections 3½ hours later they were given 10 mg/kg of morphine The analgesic effect was determined 30 minutes later

To be able to evaluate any possible effect of neostigmine on the concentration of morphine in the brain of morphine tolerant and non tolerant rats, a representative number of rats that had received morphine (10 mg/kg) alone or morphine plus neostigmine were killed immediately after the analgesic effect had been determined Their brains (including the brain stem and the cerebellum) were taken out and homogenized Morphine was determined either in brain homogenates from single animals or in pooled homogenates of brains from two animals.

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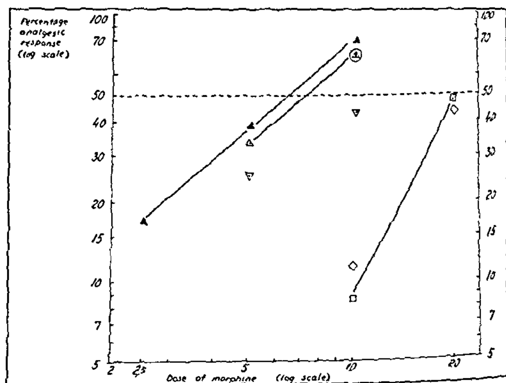


Fig 1 The analgesia produced by morphine in non-tolerant and tolerant rats with and without neostigmine (0 1 mg/kg) administration in tests performed 30 minutes after the injections The response of non-tolerant rats pretreated with reserpine is also shown *Abscissa* Dosage of morphine, log scale *Ordinate* Percentage of animals reacting analgesic (log scale).

▲—▲ NONTOLERANT, PREVIOUS RESULTS
△—△ NONTOLERANT
▽—▽ NONTOLERANT WITH NEOSTIGMINE

□—□ TOLERANT
◇—◇ TOLERANT WITH NEOSTIGMINE
○ RESERPINE PRETREATED

Results

Measurement of degree of analgesia The analgesic effect of morphine was, as could be expected, found to be significantly greater in non-tolerant than in tolerant rats (fig. 1) At the ED50 level the potency factor was 3. The reliability of the procedure is demonstrated in the figure by the inclusion of results from another investigation (JÓHANNESSON & SCHOU 1963) in which the effect of morphine on non-tolerant rats was determined

by the same method at three different dosage levels (2 5, 5 and 10 mg/kg) In the series of experiments reported here we used only two dosage levels (5 and 10 mg/kg) of morphine on non tolerant rats, and the results do not differ to any significant degree from those previously obtained

In non tolerant rats neostigmine (0 1 mg/kg) given intraperitoneally immediately after the morphine injection seemed at first sight to decrease the analgesic effect of morphine (fig 1) This result did not, however, appear significant on analysis We found $\chi^2 = 0\ 139$ at the 5 mg/kg dosage level, corresponding to P about 0 7 At the 10 mg/kg morphine level we found $\chi^2 = 4\ 10$, corresponding to $0\ 05 > P > 0\ 025$ This gives some evidence of a decrease in the analgesic effect of morphine at this dosage resulting from the simultaneous injection of neostigmine in non tolerant rats However, because of the small probability of a difference in analgesic response at the 5 mg/kg morphine level caused by neostigmine, and the relatively small probability of a difference at the 10 mg/kg morphine level we do not believe that there is a true effect of neostigmine on the analgesic response to morphine by non-tolerant rats This view is supported by the experimental results obtained on tolerant animals Here the analgesic response to morphine was the same whether the drug was given alone or with neostigmine (fig 1)

Table 1

Concentrations of morphine in brains (μg/g) of rats given morphine (10 mg/kg) alone or combined [illegible]

[illegible] analysis

a animals showing analgesia
n a animals not showing analgesia

	Non tolerant controls		Morphine tolerant	
	n a	a	n–a	a
Morphine 10 mg/kg	0 53 (1) 0 60 (1) 0 71 (1)	0 50 (1) 0 64 (2) 0 64 (2) 0 74 (1)	0 38 (1) 0 38 (2) 0 68 (2) 1 07 (1)	0 80 (2)
Morphine 10 mg/kg + neostigmine 0 1 mg/kg	0 41 (2) 0 48 (1) 1 25 (2)	n m (1) 0 36 (2) 0 88 (1)	n d. (1) 0 46 (2) 0 54 (1) 0 54 (1) 0 91 (2)	0 43 (2)

A single group of rats was pretreated with reserpine (1 mg/kg) 3½ hours before injection of 10 mg/kg morphine The analgesic responses did not differ from those of non-tolerant controls

Brain concentration of morphine To elucidate whether the penetration of morphine into the brain is altered by development of tolerance or treat ment with neostigmine, 10 mg/kg morphine was given to tolerant and non-tolerant rats with and without simultaneous neostigmine injections The animals were killed 30 minutes later, immediately after their anal gesic responses had been tested, and the brains vere removed for quanti tative morphine analysis The results are tabulated in table 1 By the t-test no significant differences were demonstrated between any of the experimental groups (P > 0 1 in all instances) The mean concentrations with their standard errors, were for the non-tolerant 0 62 ± 0 04 and for the tolerant rats 0 66 ± 0 13 When neostigmine (0 1 mg/kg) was given simultaneously with morphine, the figures for non tolerant were 0 58 ± 0 17, and 0 48 ± 0 12 for the tolerant rats If all the results from the brains of animals given neostigmine were pooled (non-tolerant and tolerant) and tested against the pooled figures for tolerant and non tolerant rats to which only morphine had been given, there was still no statistical difference between the groups (t–test, P > 0 1)

Discussion

In these experiments morphine was found to be approximately 3 times less active in the tolerant than in the non-tolerant rats at the ED50 level (cf fig 1) Almost identical amounts of morphine were found in the brains of tolerant and of non tolerant rats given the same dose (table 1) The lower analgesic effect of morphine in the tolerant rats is therefore not due to smaller amounts of the drug in the whole brain

We do not therefore believe that the concentration of diffusible morphine in the blood or the permeability of the blood-brain barrier is altered during the development of morphine tolerance We cannot draw any further conclusions about the pattern of distribution of morphine in tolerant animals from the experiments described here However, if such significant changes had been present, they would be expected to effect the concentration of morphine in the brain In studying analgesic effects it is only a rough guide to determine concentrations in the whole brain We must admit that any alteration in local concentrations in specific areas of the central nervous system owing to tolerance would not be unveiled by our experiments The sensitivity of our procedure of analysis is not sufficient for further elucidation of the distribution pattern within the

central nervous system This was recently attempted in dog by MULE & WOODS (1962) and MULE, WOODS & MELLET (1962) They used N-^{14}C-methyl labeled morphine in an investigation of the distribution pattern in the brains of tolerant and non tolerant dogs

JÓHANNESSON (1962b) administered large amounts of morphine with or without neostigmine to morphine tolerant and non tolerant rats by simultaneous intraperitoneal injections Neostigmine increased morphine [illegible]

[illegible]

other effect than an increase of morphine concentration in the brain In the experiments reported here neostigmine was, however, found not to increase the analgesic effect of morphine in morphine tolerant or non-tolerant rats This investigation has therefore confirmed that the concentration of morphine in the brains of tolerant and non tolerant rats is the same, regardless of whether the rats have received morphine alone or simultaneous with neostigmine (table 1)

Instead of a potentiation of morphine action, we found, on the contrary, that neostigmine seemed to suppress morphine analgesia to some extent in non tolerant rats However, statistical analysis showed this difference to be probably not real Apparently neostigmine had no influence upon analgesia in the toler[illegible] fore in agreement w[illegible] from the Lexingt[illegible] (1954) in experiments on guinea pigs and of HERKEN *et al* (1957) working with rats

Besides neostigmine such cholinergic substances as physostigmine and carbacholine have been found to increase the analgesic effect of morphine and pethidine (e g PÓRSZASZ *et al* 1951) In this connection it is of interest that BERTLER *et al* (1958) and VARAGIĆ & VOJVODIĆ (1962) found carbacholine and physostigmine to release catecholamines, and SCHAUMANN (1958) put forward the hypothesis that morphine analgesia is mediated through release of noradrenaline In his experiments with mice Schaumann found that pretreatment with reserpine, which depletes the tissues of 5 hydroxytryptamine and noradrenaline, antagonizes the analgesic action of morphine We were, however, not able to confirm his results in our experiments (fig 1) It is therefore concluded that morphine analgesia in the rat is not primarily connected with release of catecholamines and 5 hydroxytryptamine This view is supported by recent experiments by SLOAN *et al* (1963) They found identical concentrations of catecholamines and 5 hydroxytryptamine in brains of non tolerant rats and

highly tolerant animals killed 24 hours after the last morphine injection If the cholinergic substances mentioned above do exert any influence at all on morphine analgesia in the rat, it is at any rate not connected with release of these biologically active amines.

Summary

A method for measuring degree of analgesia has been used to compare the analgesic potency of morphine in tolerant and non-tolerant rats At the ED50 level, morphine was about 3 times more potent in non-tolerant than in tolerant animals. Neither in non-tolerant nor in tolerant rats did intraperitoneal injection of neostigmine alter the analgesic response to any significant degree.

The concentration of morphine found in the brains 30 minutes after intraperitoneal injection of 10 mg/kg morphine did not differ significantly between tolerant and non-tolerant rats. Intraperitoneal injection of neostigmine (0.1 mg/kg) simultaneously with the morphine injection did not influence the concentrations of morphine in the brain. Pretreatment with reserpine (1 mg/kg) did not alter the analgesic response to morphine in non-tolerant rats.

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REFERENCES

Andrews, H L The effect of morphine and prostigmine methylsulfate on measurements of pain threshold *J A M A* 1942, 120, 525–526

Bertler, Å, A Carlsson, M Lindqvist & T Magnusson On the catechol amine levels in blood plasma after stimulation of the sympathoadrenal system *Experientia* 1958, 14, 184–185

Burn, J. H, D J Finney & L G Goodwin *Biological standardization* Oxford University Press, 1950 (p 109)

Christensen, E M & E G Gro[illegible] morphine, meperidine and m[illegible]

Flodmark, S & Th Wrammer [illegible] and prostigmine studied by [illegible] *Acta physiol Scandinav.* 1945, 9, 88–96

Herken, H, D Maibauer & S Muller Acetylcholingehalt des Gehirns und [illegible]

[illegible]

Jóhannesson[illegible] morphine tolerant and non-tolerant rats *Acta pharmacol et toxicol* 1962a, 19, 23–35

Jóhannesson, T The concentrations of morphine in the brains of rats receiving morphine and neostigmine *Acta pharmacol et toxicol* 1962b, 19, 286–292

Jóhannesson, T & K. Milthers· Determination of morphine *Radiometer News* 1963 (in press)

Jóhannesson, T & J Schou Morphine and normorphine in the brain of rats given identically analgaesic doses of morphine, codeine or normorphine *Acta pharmacol et toxicol* 1963, 20, 165–173.

Knoll, J , E. Komlós & & L Tardos Über die Rolle der Eiweissbindung im [illegible]

[illegible]

Mulé, S J & L A Woods Distribution of N [14]C-methyl labeled morphine I In central nervous system of non tolerant and tolerant dogs *J Pharmacol* 1962, 136, 232–241

Mulé, S J , L. A Woods & L B Mellet Distribution of N-[14]C-methyl labeled morphine II Effect of nalorphine in the central nervous system of non tolerant dogs and observations on metabolism *J Pharmacol* 1962, 136, 242–249

[illegible]

Scha [illegible] -chanismus [illegible] -240

Slau [illegible]

Sloan J W J W Brooks [illegible] The effect of addictic [illegible] tissue catechol-amine [illegible] 3, 4, 261–270

Szerb, J C [illegible] levallor [illegible] morphine and

Szerb, J [illegible] brain [illegible] and n [illegible]

Varagić, V [illegible] mate [illegible] *J Ph* [illegible]

Woods, L [illegible] morphine in non tolerant and tolerant dogs and rats *J Pharmacol* 1954, 112, 158–175

Acta pharmacol et toxicol 20, 222–226

From the Research Laboratory of H Lundbeck & Co A/S,
Ottiliavej 7, Copenhagen, Denmark

Hydroaminacrine II.
Its Effect on the Interaction of Edrophonium and Decamethonium

By

V. Dyrberg*), W. Hougs and S H. Johansen*)

(Received August 8 1963)

Hydroaminacrine, which is a powerful inhibitor of plasma cholinesterase (SHAW & BENTLEY 1953, JENSEN HOLM *et al* 1961), reduces the amount of suxamethonium necessary to produce neuromuscular blockade in the rabbit by 60–70% (DYRBERG *et al* 1962) This reduction is most probably related to the fact that suxamethonium is hydrolysed by the cholinesterase of the plasma In the experiments reported here, decamethonium was included for purposes of comparison, since the degree of muscular paralysis with this agent is not affected by changes in the plasma content of this enzyme

Technique

As in our investigation into the inter relationship between hydroaminacrine and suxamethonium we have regularly used in the rabbit repeated intravenous injections of edrophonium and have taken antagonism developed on the indirectly stimulated tibial muscle as indication of the occurrence of dual block

The experimental technique has been described previously (DYRBERG *et al* 1962) Ten rabbits were used of which 5 received hydroaminacrine in a single intravenous dose of 1 mg per kg

Decamethonium (synacur ®) was infused into the vein at a concentration of 0 1 mg per ml

Edrophonium (tensilon ®) 0 2 mg per kg was injected intravenously every 15 minutes

Results

As during the infusion of suxamethonium, edrophonium produced in the presence of decamethonium different responses, which followed a

*) Department of Anaesthesia Copenhagen County Hospital in Gentofte Hellerup Denmark

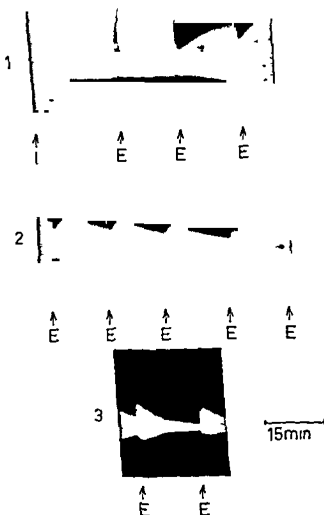


Fig 1 Rabbit (3 2 kg) chloralose (43 mg per kg) urethane (430 mg per kg) Indirectly supramaximal stimulation of the anterior tibial muscle Kymographic recording during continuous intravenous infusion of decamethonium
Every 15 minutes edrophonium 0 2 mg per kg injected intravenously, (E)
1 start of infusion (I)
2 90 min after start of infusion
3 240 min after start of infusion

certain pattern, the normal sequence was antagonistic, biphasic, synergistic and finally antagonistic (fig 1) In two animals untreated with hydroaminacrine no initial antagonism was observed

There was no difference between the two groups of animals as to when

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RIKER *et al* (1957, 1959) described the biphasic response of these drugs, lemonstrating that simultaneously with the facilitatory action a depolari-ation of the end plate region may take place, especially with the metho-nium ions, which would lead to a block of depolarization The facilita-ion which can be observed also with acetylcholine, is of short duration with edrophonium (NASTUK & ALEXANDER 1954)

Consequently, the first two phases in the interaction of decamethonium and edrophonium demonstrate the biphasic response of the latter, whereas the final antagonism demonstrates the modification of the decamethonium-induced depolarization block that occurs with time, viz. the dual block (ZAIMIS 1953) As with suxamethonium (DYRBERG *et al* 1962), hydro-aminacrine did not alter the time of occurrence of the dual block with decamethonium

Neither did hydroaminacrine modify the amounts of decamethonium necessary to maintain the myoneural block This points to the fact that hydroaminacrine is an inhibitor predominantly of serum cholinesterase, which plays no part in the elimination of decamethonium (PATON & ZAIMIS 1952)

Unequivocal signs of facilitation were not observed when edrophonium was administered in the presence of suxamethonium, which suggests that suxamethonium already had triggered off the facilitation response, and confirms the observation of NASTUK & ALEXANDER (1954) that the facilitation effect is evanescent with certain drugs

Hydroaminacrine, given after the infusion of decamethonium had begun produced a weak increase in twitch tension, which could be taken as a sign that this agent also facilitated myoneural transmission

Thus hydroaminacrine is without influence on the time of onset of dual block defined as developed antagonism to edrophonium during myo-neural blockade with suxamethonium (DYRBERG *et al* 1962) and decame thonium and appears to have a limited influence on neuromuscular blockade with depolarizing substances Since it has a moderate anta gonistic effect towards the non depolarizing agents (SHAW & BENTLEY [illegible]

[illegible] terminals to facilitate transmission without affecting the end plate potentials

Summary

In two groups of rabbits one of which was treated with hydroamina- [illegible]

Table 1

Amounts of decamethonium infused at occurrence of antagonism to edrophonium in five rabbits pretreated with hydroaminacrine and five controls

	Decamethonium mg/kg	Duration minutes	mg/kg/min
Hydroaminacrine 1 mg/kg i v	1 8	80	0 023
	2 7	105	0 026
	3 3	150	0 022
	2 8	105	0 027
	1 6	60	0 027
Average	2 4 ± 0 3	100 ± 15	0 025 ± 0 001
Controls	2 6	90	0 029
	0 8	35	0 023
	2 1	120	0 018
	2 4	105	0 023
	2 7	150	0 018
Average	2 1 ± 0 3	100 ± 18	0 022 ± 0 001

the final antagonism developed and how much decamethonium had been infused to produce this reaction (table 1) In three of the five animals given hydroaminacrine, this was injected after the infusion of decamethonium had begun, in two of these a moderate antagonism to the neuromuscular block ensued, whereas no response was shown by the third

Discussion

The four-phasic response, which edrophonium produces during myoneural blockade with decamethonium, was not affected by hydroaminacrine The antagonistic reaction after the first dose of edrophonium was often pronounced, restoring the twitch tension to nearly normal levels, but subsequent injections gave increasingly weaker responses and were accompanied by muscular depression This brief antagonism is probably indicative of the facilitation of neuromuscular transmission occurring with edrophonium and other quaternary ammonium salts, it has been analysed by NASTUK & ALEXANDER (1954) and RIKER *et al* (1957) The facilitation was shown to be independent of end-plate depolarization and has been attributed to the phenomenon that these drugs, through action on the motor-nerve terminal, change the response to single stimuli into a multiple repetitive discharge simulating a tetanic stimulation

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From the Department of Medical Chemistry, University of Turku,
Turku 3, Finland.

Effect of Lathyrism on Nucleic Acids and Subcellular Particles of the Experimental Granulation Tissue

By

H. Isomäki and E. Kulonen

(Received August 17, 1963)

In earlier studies we have observed that in lathyrism (an intoxication caused by β aminopropionitrile or aminoacetonitrile) the incorporation of amino acids in collagen was decreased (KULONEN, SALMI & JUVA 1960), the total concentration of nitrogenous substances in the tissue was decreased (VILJANTO, ISOMÄKI & KULONEN 1962), and the incorporation of amino acids into protein was retarded (SALMI & KULONEN 1962) It is also known (VILJANTO unpublished work) that in the granulation tissue the amount of RNA and the number of microsomes increase in parallel with the amount of collagen This prompted a study of the effect of lathyrism on subcellular particles and the nucleic acids in experimental granuloma

Experimental

Treatment of the animals In the preliminary series 40 rats received daily each 40 mg aminoacetonitrile (AAN) beginning 10 days before the implantation of the cellulose sponges (VILJANTO & KULONEN 1962) The same number of rats served as controls The food was restricted to about a half the normal consumption The animals conse [illegible]

[illegible] rats was zero in the AAN group but in the control
gro[illegible] the [illegible]

(45 [illegible]
da[illegible]
tio [illegible]
tio [illegible] in this group

Edriphonium produced in both groups a four-phasic response: antagonistic, biphasic, synergistic and finally antagonistic.

Hydroaminacrine did not increase the effect of decamethonium and did not alter the time needed for the final antagonism between edrophonium and decamethonium to develop.

REFERENCES

Dyrberg, V, W. Hougs & S H Johansen: Hydroaminacrine I – Its Influence on the Interaction of Edrophonium and Suxamethonium *Acta pharmacol et toxicol* 1962, 19, 68–72

Jensen-Holm, J, K. Stubbe Teglbjerg & W. Hougs The Effect of Hydro- [illegible] Isolated Guinea [illegible]

Nast [illegible]

J. Pharmacol 1954, 111, 302–328

Paton, W D M & E J. Zaimis. Methonium Compounds *Pharmacol Rev* 1952, 4, 219–249.

Rike [illegible] The Motor Nerve [illegible]

Rike [illegible] Element in Neuromuscular Transmission *Ann New York Acad Sci* 1959, 81, 328–344

Shaw, F H & G Bentley The Pharmacology of some New Anti-Cholinesterases *Austral J Exp Biol* 1953, 31, 573–576

Zaimis, E. J Motor End Plate Differences as a Determining Factor in the Mode of Action of Neuromuscular Blocking Substances *J Pharmacol* 1953, 122, 238–251

0 75 N sulphuric acid and extracted with ether to remove the trichloroacetic acid [illegible] The val[illegible]e of RNA was [illegible] ic washing fluids The same source of error has been observed also by HALLINAN, FLECK & MUNRO (1963) The organic washings were evaporated to about 5 ml and made alkaline with 0 1 N sodium hydroxide TCA was added to a concentration of 5%, and the solutions were heated at +90°C for 15 min The nucleic acids were determined in this extract as described above, and the necessary correction was added to the results This addition was largest for the RNA values of the 10th day sample (0 17 mg/cm³ for AAN-samples and 0 53 mg/cm³ for normal samples)

The TCA-soluble nucleotides were also determined. The total ribonucleotide content of 10th day granulomas in the samples from AAN treated animals was about half that of control animals

Nitrogen and *hydroxyproline* were both determined after hydrolysis of the samples overnight at +100°C in closed tubes in 6 N HCl Nitrogen was determined by a micro-Kjeldahl procedure and hydroxyproline by that of NEUMAN & LOGAN (1950)

Table 1

Subcellular particles in granulation tissue*)

Description		5th day granuloma		10th day granuloma	
		control	AAN treated	control	AAN treated
Weight of the animals g					
initial average		102	102	102	102
final average		120	116	121	117
increase rat/day		1 50	1 16	1 12	0 88
Hemoglobin in the granuloma mg cm³					
range		0 86–1 42	0 75–1 37	1 80–2 45	1 63–1 84
average		1 19	0 98	2 10	1 75
Nuclei and debris					
nitrogen	range	0 110–0 252	0 077–0 144	0 146–0 314	0 073–0 185
mg cm³	average	0 184	0 112	0 219	0 140
hydroxyproline	range	1 05–2 40	1 10–1 70	2 80–13 95	2 50–6 80
µg cm³	average	1 87	1 35	7.38	4 83
Mitochondrial fraction					
nitrogen	range	0 086–0 155	0 032–0 085	0 161–0 220	0 096–0 193
mg cm³	average	0 117	0 060	0 187	0 132
hydroxyproline	range	0 80–1 40	0 60–1 35	1 40–2.35	1 00–1 70
µg cm³	average	1 12	0 97	1 97	1.33
[illegible]	[illegible]	[illegible]	0 087–0 138	0 259–0 348	0 183–0 221
[illegible]	[illegible]	[illegible]	0 112	0 315	0 198
[illegible]	[illegible]	[illegible]15–1 25	0 90–1 30	1 65–2 80	1 50–2.35
µg cm³	average	1 18	1 15	2.35	1 90

*) Animals of the main series only 25 mg AAN daily Three samples of six granulomas each were analyzed

The aminoacetonitrile was available as hydrosulphate (Abbot Laboratories, Chicago, gift of Dr. A van den Hooff), which was dissolved in water, the solution (2 ml) was mixed with the usual food paste

Before the implantation, 10–15 rats were kept in one cage, after implantation tw rats lived in same cage with steel wire floor

Implantation The "Visella"-sponges (of viscose cellulose) were implanted previously described (VILJANTO & KULONEN 1962, VILJANTO, ISOMÄKI & KULONI 1962) The weight of the pieces in the main series was 72 6 ± 2 3 mg (air-dried) ar the size 10 × 10 × 20 mm = 2 cm^3 (wet)

The rats were killed by ether 5 or 10 days after the implantation, and the spong with their granulation tissue were extracted The pieces were prepared free from tl adjoining surface tissue, and the granulomas were stored frozen at −18°C.

Fractionation of the subcellular particles Six granulomas were combined into sample Three samples were obtained and analysed independently

The pieces were homogenized (in a Buhler homogenizer, 50000 r p m) into 40 r of 0 9% (w/v) NaCl solution and allowed to flow through cheese-cloth to remove tl coarse debris and parts of the sponge, which were washed with 20 ml of the same NaC solution The homogenate was centrifuged at +4°C for 10 min at 600 G in an MS refrigerated centrifuge The sediment contained the fine debris and nuclei It w washed twice with the NaCl solution, and the washings were similarly centrifuged 600 G From the combined supernatants the mitochondrial fraction was obtained t 15 min centrifugation at 5000 G The sediment was washed five times with NaC solution, the washings were centrifuged at 6600 G and the supernatants were combi ed The microsomal fraction was obtained by centrifugation for 90 min at 35000 C it was washed once with NaCl solution The procedure was that of BOUCEK, NOBI & WOESSNER (1959)

The sediments were washed into test tubes with 2 ml of NaCl solution and store frozen at −18°C

Analyses Haemoglobin was determined as cyanmethaemoglobin at the wave lengt of 5400 Å in the supernatant from separation of the microsomal fraction To 1 ml the supernatant were added 2 ml of the reagent containing 1% (w/v) potassiu ferricyanide and 0 5% (w/v) sodium cyanide For the values of molar extinction 11 and of the molecular weight 16520 were accepted The result was calculated as mg haemoglobin/cm^3 of granulation tissue

The *dry weight* was determined after drying the pieces of granuloma for three da at +105°C The net weight was obtained by subtraction

For determining *nucleic acids* six granulomas (12 cm^3) were pooled (thre samples) The pieces of tissue were homogenized into 2½ volumes of 0 9% NaCl solu tion at the maximum speed of the Bühler homogenizer The homogenate was coole to 0–1°C and an equal volume of 10% (w/v) cold trichloroacetic acid was addec [illegible] The sediment wa

suspended i

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of 96% (w/v

e times wit

20 ml of 5% (w/v) trichloroacetic acid for 30 min at +90°C The extracts were com bined and their volume made up to 60 ml with 5% trichloroacetic acid The extractio procedure was a modification of that described by BIGGERS LAWSON, LUCY & WEBI (1961)

The DNA was determined in the combined extracts by the diphenylamine reactior of BURTON (1956) For estimation of total nucleic acids the extract was adjusted tc

The amounts of both DNA and RNA are low in the granulomas of lathyritic animals, indeed the decrease is larger than the decrease in haemoglobin in the same granulomas The effect is more marked in 10th day than in 5th day granulation tissue From the ratios RNA/DNA it is evident that the synthesis of ribonucleic acid is especially retarded, the normal steep rise between the 5th and 10th days is moderate in the granulomas of lathyritic rats

Discussion

More information on the RNA fractions is necessary for evaluating the mechanism and significance of defective nucleic acid synthesis It is as yet not possible to correlate the disturbance in nucleic acid synthesis with the macromolecular maturation defect of collagen, which is the characteristic feature in lathyrism (NIKKARI & KULONEN 1962)

Summary

The content of nucleic acids (especially of RNA) was greatly decreased in the experimental granulation tissue of lathyritic rats This diminution was more marked than the reduction observed in the weight gain of the rats or in the development of capillary circulation in the granulation tissue The absolute amounts of the mitochondrial and microsomal nitrogen decreased also, but to a less extent The total hydroxyproline was fairly high in the microsomal fractions of granulomas of lathyritic rats

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REFERENCES

Biggers, J D K A Lawson J A Lucy & M Webb The chemical composition of long bone rudiments from the embryonic chick *Biochim Biophys Acta* 1961 54 236–248

Boucek R J N L Noble & J F Woessner Jr The effects of tissue age and sex upon connective tissue metabolism *Annals New York Academy of Sciences* 1959 72 1016–1030

Burton K A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid *Biochem J* 1956 62 315 323

Results

Only the results of the second main series are given here in detail Weight gain and vascularisation (as reflected by the haemoglobin content) are decreased in lathyritic animals, but the difference does not seem so large, as to make the granulation tissue non-viable (table 1). In the sub cellular particles the effect of lathyrism is much more marked The nuclear fraction may not be of great significance, because it contains much debris of undefined nature, but in the mitochondrial and microsomal fractions the decrease in nitrogen content seems genuine and is not due to contamination with small fragments of collagen. When the microsomal nitrogen is calculated on the basis of total DNA, *there is no change* due to lathyrism (table 2). The total amount of hydroxyproline bound to the subcellular fractions is little affected by the intoxication. On the contrary, the amount of hydroxyproline in the microsomal fraction, calculated on the basis of total DNA, total RNA or microsomal nitrogen (table 2), is increased. Caution is necessary, however, because in lathyrism the collagen may be present in such small fragments that they sediment at the same rate as microsomes

Table 2.

Nucleic acids in granulation tissue*)

Description		5th day granuloma		10th day granuloma	
		control	AAN-treated*)	control	AAN treated*)
DNA, mg/cm^3	range	1 01–1 07	0 57–1 16	1 31–1 73	0 75–1 07
	average	1 03	0 88	1 53	0 84
RNA, mg/cm^3	range	0 25–0 45	0 19–0 31	2 18–2 38	0 52–0 73
	average	0 34	0 24	2 28	0 60
RNA/DNA	range	0 25–0 42	0 25–0 33	1 38–1 66	0 68–0 74
	average	0 33	0 28	1 51	0 70
Microsomal N/tot DNA, mg/mg	range	0 104–0 154	0 119–0 153	0 198–0 218	0 206–0 259
	average	0 134	0 132	0 206	0 236
Microsomal OP/tot DNA, µg/mg	range	1 13–1 17	1 03–2 19	1 26–1 68	2 05–2 47
	average	1 14	1 45	1.52	2 24
Microsomal OP/tot RNA, µg/mg	range	2 74–4 60	4 19–5 68	0 76–1 18	2 88–3 43
	average	3 65	4 87	1 02	3 18
Microsomal OP/N, µg/mg	range	7 37–10 84	9 42–11 36	6 37–8 05	8 20–10 63
	average	8 75	10 37	7 37	9 54

*) Animals of the main series only, 25 mg AAN daily Three samples of six granulomas each were analyzed

The amounts of both DNA and RNA are low in the granulomas of lathyritic animals, indeed the decrease is larger than the decrease in haemoglobin in the same granulomas The effect is more marked in 10th day than in 5th day granulation tissue From the ratios RNA/DNA it is evident that the synthesis of ribonucleic acid is especially retarded, the normal steep rise between the 5th and 10th days is moderate in the granulomas of lathyritic rats

Discussion

More information on the RNA fractions is necessary for evaluating the mechanism and significance of defective nucleic acid synthesis It is as yet not possible to correlate the disturbance in nucleic acid synthesis with the macromolecular maturation defect of collagen, which is the characteristic feature in lathyrism (NIKKARI & KULONEN 1962)

Summary

The content of nucleic acids (especially of RNA) was greatly decreased in the experimental granulation tissue of lathyritic rats This diminution was more marked than the reduction observed in the weight gain of the rats or in the development of capillary circulation in the granulation tissue The absolute amounts of the mitochondrial and microsomal nitrogen decreased also but to a less extent The total hydroxyproline was fairly high in the microsomal fractions of granulomas of lathyritic rats

Acknowledgements

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REFERENCES

Biggers J D K A Lawson J A Lucy & M Webb The chemical composition of long bone rudiments from the embryonic chick *Biochim Biophys Acta* 1961 54 236–248

Boucek R J N L Noble & J F Woessner Jr The effects of tissue age and sex upon connective tissue metabolism *Annals New York Academy of Sciences* 1959 72 1016–1030

Burton K A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid *Biochem J* 1956 62 315 323

Results

Only the results of the second main series are given here in detail. Weight gain and vascularisation (as reflected by the haemoglobin content) are decreased in lathyritic animals, but the difference does not seem so large, as to make the granulation tissue non-viable (table 1). In the subcellular particles the effect of lathyrism is much more marked. The nuclear fraction may not be of great significance, because it contains much debris of undefined nature, but in the mitochondrial and microsomal fractions the decrease in nitrogen content seems genuine and is not due to contamination with small fragments of collagen. When the microsomal nitrogen is calculated on the basis of total DNA, there is no change due to lathyrism (table 2). The total amount of hydroxyproline bound to the subcellular fractions is little affected by the intoxication. On the contrary, the amount of hydroxyproline in the microsomal fraction, calculated on the basis of total DNA, total RNA or microsomal nitrogen (table 2), is increased. Caution is necessary, however, because in lathyrism the collagen may be present in such small fragments that they sediment at the same rate as microsomes.

Table 2.

Nucleic acids in granulation tissue*)

Description		5th day granuloma		10th day granuloma	
		control	AAN-treated*)	control	AAN-treated*)
DNA, mg/cm³	range	1.01–1.07	0.57–1.16	1.31–1.73	0.75–1.07
	average	1.03	0.88	1.53	0.84
RNA, mg/cm³	range	0.25–0.45	0.19–0.31	2.18–2.38	0.52–0.73
	average	0.34	0.24	2.28	0.60
RNA/DNA	range	0.25–0.42	0.25–0.33	1.38–1.66	0.68–0.74
	average	0.33	0.28	1.51	0.70
Microsomal N/tot DNA, *mg/mg*	range	0.104–0.154	0.119–0.153	0.198–0.218	0.206–0.259
	average	0.134	0.132	0.206	0.236
Microsomal OP/tot DNA, µg/mg	range	1.13–1.17	1.03–2.19	1.26–1.68	2.05–2.47
	average	1.14	1.45	1.52	2.24
Microsomal OP/tot RNA, µg/mg	range	2.74–4.60	4.19–5.68	0.76–1.18	2.88–3.43
	average	3.65	4.87	1.02	3.18
Microsomal OP/N, µg/mg	range	7.37–10.84	9.42–11.36	6.37–8.05	8.20–10.63
	average	8.75	10.37	7.37	9.54

*) Animals of the main series only, 25 mg AAN daily. Three samples of six granulomas each were analyzed.

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From the Biological Department,
Royal Danish School of Pharmacy, Copenhagen

A Sensitive Method for Estimation of Ergometrine, Based Upon Serotonin Antagonism on Isolated Rat Uterus

By

Reidar Bredo Sund*)

(Received August 21, 1963)

Ergometrine in aqueous solution can undergo oxidation, epimerization, conversion to *lumi* alkaloids and hydrolysis, a variety of deterioration products may thus arise Because of the inadequacy of the usual chemical methods for specifically determining unchanged ergometrine, there is a need for a reliable biological method As is true for determinations of adrenaline, bioassays might prove to be as accurate, precise and rapid as chemical ones, which would obviously involve separations, e g. by paper chromatography

A survey of the literature reveals that many methods have been suggested for the bioassay of ergometrine These have been based upon extra-uterine effects such as cyanosis of the cock's comb (SWANSON, HARGREAVES & CHEN 1935), mydriasis in rabbits and mice (DE BEER & TULLAR 1941; VOTAVA & PODVALOVÁ 1957), hyperthermia in rabbits (DE BEER & TULLAR *1941*) or upon the oxytocic effect in various mammals, either *in vivo* or *in vitro* (SWANSON, HARGREAVES & CHEN 1935, ROTHLIN 1938, WICK & POWELL 1942, VOS 1943, FOSTER & STEWART 1948, PENNEFATHER 1961) No one of these methods, however, seems to have gained general acceptance (FOSTER & STEWART 1948) Also the recently published method of PENNEFATHER (1961), involving the oxytocic effect on isolated rat uterus, has been criticised (BERDE & STÜRMER 1962, SUND 1963) Instead, it has been suggested that determinations might be based on the antagonism to serotonin on rat uterus, after abolition of the oxytocic action by magnesium ions (SUND 1963) This suggestion has been examined practically For comparison, ergometrinine and *lumi*-ergometrine, methylergometrine and ergotamine were also included in the *investigation*

*) Present adress [illegible]

Hallinan, T, A Fleck & H N Munro Loss of ribonucleic acid into lipid solvents after acid precipitation *Biochim Biophys Acta* 1963, 68, 131–133

Kulonen, E, Annikki Salmi & K. Juva Experiments on the metabolism of collagen in lathyrism *Biochem J* 1960, 76 54P

Neuman, R E & M A Logan The determination of hydroxyproline. *J Biol Chem* 1950, 184, 299–306

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Viljanto, J, H Isomäki & E Kulonen Effect of aminoacetonitrile iproniazid and semicarbazide on the tensile strength of experimental granulation tissue *Acta pharmacol et toxicol* 1962, 19, 191–198

Viljanto, J & E. Kulonen Correlation of tensile strength and chemical composition in experimental granuloma *Acta Path Microbiol Scand* 1962, 56 120–126

maximal dose of serotonin every 4 min The height of the "twitches" first increased in a characteristic, slow stepwise manner, flattened out and gradually fell off, reaching a stable level within about one hour and a half A 6 min dose cycle was then started and 3 un inhibited contractions were recorded with the serotonin dose finally chosen

3 "Priming" with standard ergometrine From now on, the dose cycle included these operations a) Addition of antagonist, contact about 2 min b) Emptying the bath c) Refilling with serotonin, contact for 50–70 sec d) Washing in 3 successive changes of fluid e) Rest f) Emptying the bath, and repeating the cycle

4 Matching the unknown preparation to the standard When a good measure was obtained of its activity, fresh dilutions of both preparations were prepared They could be used throughout the assay

5 (2 + 2) assay according to SCHILD (1942) The ratio between doses was 1 2, and as a rule 6 randomised groups were used

6 Recording of 3 un inhibited contractions

Calculation The results were calculated as by BRISEID JENSEN & VENNEROD (1961) For comparison, calculations have also been based directly on the height of contractions themselves (see below)

Comments on Method

Contact periods and cycle time The maximum inhibitory effect of a single small dose of ergometrine is reached fairly soon Increasing the contact period to this antagonist from 2 to 8 minutes affected appreciably neither sensitivity nor precision

Further, the effect disappears rapidly In a 4 minutes' cycle, however, some inhibition persists until the next contraction A few experiments (test standard = 1 25, 6 randomised groups) have been performed with a cycle time of that length and recovery doses of serotonin alternating with direct inhibition The index of precision was 0 043–0 055 When the increase in experimental time is remembered, this procedure appears no more suitable than the final (*cf* table 1) Moreover, the latter can be performed fully automatically on commercially available instruments not used here

In a 6 minutes' cycle, rest inhibition was absent or negligible

The period of contact to serotonin should be so adjusted that the contractions have passed their maxima before the drug is washed away Otherwise, the washing might act as an extra irritant or "cut" the contractions

Calculation The calculation procedure (BRISEID JENSEN & VENNEROD

Materials

Serotonin 5-Hydroxytryptamine creatinine sulphate, Hoffman La Roche & Co Ltd, Basel, Switzerland

Ergometrine Ergometrinum Ph Dan 1948 Stock solutions (1 mg ergometrine/ml) consisting of equimolar amounts of ergometrine and d-tartaric acid (pH about 3 6) were stable for at least 3 months when kept in the dark at +4° and assayed by the method described below

*Ergometrinine**) Ergobasinine, Sandoz A G, Basel, Switzerland

lumi-Ergometrine I (β)*) *lumi*-Ergobasine, Sandoz A G

Methylergometrine Methergin ® amp, Sandoz A G

Ergotamine Gynergen ® amp, Sandoz A G

Bathing fluid This was a modified Beauvillain solution (BEAUVILLAIN 1943), containing 9 0 g NaCl, 0 42 g KCl, 0 096 g $MgCl_2$ (1 mM, *cf* SUND 1963), 0 06 g $CaCl_2$, 0 5 g $NaHCO_3$ and 0 5 g glucose per litre In a few experiments only 0 5 mM magnesium chloride was used, and in one as much as 6 mM

Experimental dilutions Doses refer to 5-HT creatinine sulphate and to the free bases of the antagonists and are expressed as the concentrations (ng/ml) of final experimental dilutions prepared in the bath fluid The contractions to be inhibited were 60% or more of the maximal, requiring serotonin doses of 30–100 ng/ml Ergometrine doses were usually in the range 2 0–8 0 ng/ml All solutions of ergot alkaloids were protected against light

Method

Uterus The mid section (about 3 cm) of freshly dissected uterine horns from 3–5 months old virgin albino rats in spontaneous oestrus were attached in a 3 ml bath to an isotonic frontal lever (SCHILD 1947) magnifying 4-fold on a smoked drum, usually a load of 1 1–1 25 g was applied to the organ

Apparatus The assays were performed on an apparatus similar to that described by BOURA, MONGAR & SCHILD (1954), which was modified for four antagonist dilutions as described by BRISEID JENSEN & VENNEROD (1961) This means that the change over from one antagonist dilution to another had to be made by hand, but all other operations could be automatically performed

Procedure This consisted of six steps

1 Relaxation of uterus for about half an hour
2 Stabilization period The uterus was stimulated by a suitable sub

* Kindly furnished by Dr A Hofmann Sandoz A G

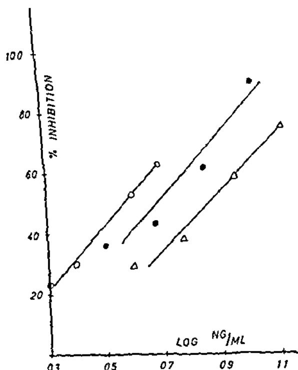


Fig 1 Ergometrine log-dose response curves obtained from 3 different uteri Each dose was given 4 times

Table 1.

Accuracy and precision of the method

Ergometrine solutions (11) of strengths unknown to the operator were assayed on different uteri. s/b = "index of precision" Further experimental details are contained in tables 2 and 3

Assay no	Magnesium chloride concentration, mM	Ergometrine µg/ml Theoretical	Ergometrine µg/ml Found	Departure from theoretical %	s/b	Fiducial limits % (P = 0 05)
1	0 5	6 00	5 65	−5 8	0 068	87–115
2		1 15	1 25	+8 6	0 056	89–112
3		1 50	1 40	−6 7	0 039	92–108
4		5 50	5 33	−3 1	0 080	85–117
5	1 0	2 50	2.26	−9 6	0 059	89–113
6*		3 80	3 64	−4 2	0 078	82–123
7		2.50	2 28	−8 8	0 052	90–111
8		3 00	3 06	+2.0	0 074	85–117
9		1 00	0 99	−1 0	0 050	91–110
10		2.00	2.14	+7 0	0 062	88–113
11	6 0	10 0	10 3	+3 0	0 080	85–117

* Only 4 groups given

1961) aims at correcting the individual figures of the assay for variation due to systematic changes in sensitivity to serotonin It is based upon the assumption that the non inhibited responses vary lineary with time throughout the experiment This need not be true over a fairly long experimental period, and over-correction as well as under correction could result Ideally, too, the changes should not be so great that the linear range of the serotonin log dose response curve is surpassed This may happen when the sensitivity increases if the standard serotonin dose at the beginning of the assay causes a fairly large effect

The experimental design *itself* largely eliminates influence in *the estimated potency* from sensitivity changes Moreover, by means of the statistical treatment (SCHILD 1942), variation resulting from such changes is sub tracted before *the estimate of error* is made Thus, calculation directly from the kymograph tracings might prove equally suitable For comparison son, this less elaborate method has been tried in the experiments showing the greatest positive and negative changes The results corresponded closely to those in table 1 Also, the differences were in both directions The corrected inhibition percentages were maintained mainly because they express in a plain way the effect of the antagonist (fig 1 and table 3)

Results

Ergometrine Fig 1 shows log dose response curves obtained from 3 rats and drawn as straight lines

11 solutions of strengths unknown to the operator have been assayed (table 1) Mean departure from theoretical activity was −1 7% (range −9 6 — +8 6), harmonic mean of 'index of precision" (GADDUM 1953) 0 060 From 6 randomised groups the resulting fiducial limits were from 85–117 to 92–108%, which can be considered satisfactory Example of an assay (no 9, table 1) is shown in fig 2 and further detail from the assays in tables 2 and 3

In assay no 8 a slightly significant departure from parallelism of regression lines was observed (0 05 > P > 0 01) This may happen by chance, but might also be due to curvature in the log dose response curves Even if so, the assay is not invalidated, provided the *fundamental* curves for the standard and test preparation are superposable (*cf* GRIDGEMAN 1944 and others), which must be true here The estimate of error was made without allowing for any departure from parallelism

Ergometrinine and lumi ergometrine The epimer, ergometrinine, and the main irradiation product, *lumi* ergometrine I or β (STOLL & SCHLIENTZ 1955, HELLBERG 1957), behaved qualitatively like ergometrine, but were

Table 3.

Results from the 11 ergometrine assays (table 1)

Assay no	Serotonin: Un inhibited response, mm: Before assay	Serotonin: Un inhibited response, mm: After assay	Serotonin: Average change in response, ±mm	Serotonin: Maximal effect, mm	Ergometrine Average inhibition, %: With lowest dose	Ergometrine Average inhibition, %: With highest dose
1	45	36	−0 20	52	32 8	65 8
2	44	40	−0 13	56	25 6	41 0
3	38	29	−0 36	42	19 5	70 0
4	44	35	−0 26	53	19 0	32 3
5	50	39	−0 28	69	18 3	57 0
6	33	36	+0 09	41	16 7	41 0
7	35	32	−0 10	52	21 5	53 8
8	39	44	+0 14	63	24 1	45 3
9	33	32	−0 03	51	22 5	47 8
10	36	39	+0 10	61	21 7	54 5
11	43	30	−0 33	not recorded	9 0	29 8

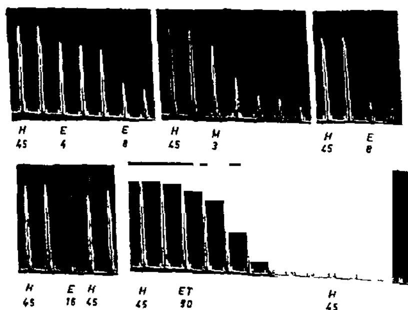


Fig 3



Fig 2 Example of ergometrine assay (no 9 table 1) The 2 middle groups of a total of 6 have been omitted from the record The drum was run from just before ergometrine was added until the serotonin was washed away
R Recovery doses of serotonin (60 ng/ml)
E Priming doses of ergometrine (4 ng/ml)
A and B Inhibition produced by the test preparat on diluted to 2 0 and 4 0 ng/ml respectively
S_1 and S_2 Standard ergometrine 4 0 and 8 0 ng/ml
T_1 and T_2 Test ergometrine 4 0 and 8 0 ng/ml

much less potent Their activities relative to ergometrine were 1 9 and 0 8% respectively These small figures may be due to slight contamination with ergometrine, but ergometrinine anyhow is held to be itself slightly active (*cf* BARGER 1938, WHITE 1944)

By the method of Vos (1943) ergometrinine also had only a small relative activity but in equimolar mixture with ergometrine it potentiated the latter This was observed neither with ergometrinine nor with *lumi* ergometrine in the present experiments

Table 2

Dose and load values from the 11 ergometrine assays (table 1)

Assay no	Load g	Serotonin ng/ml	Serotonin Contact period sec	Ergometrine ng/ml Standard doses	Ergometrine ng/ml Test doses
1	1 1	50	66	2 0 4 0	2 4 4 8
2	1 25	80	62	1 5 3 0	1 44 2 88
3	1 1	30	71	2 0 4 0	2 0 4 0
4	1 25	100	57	4 0 8 0	4 13 8 26
5	1 1	40	59	4 0 8 0	3 75 7 5
6	1 1	80	55	6 0 12 0	5 7 11 4
7	1 25	40	54	4 0 8 0	3 75 7 5
8	1 5	80		2 0– 4 0	1 5 3 0
9	1 25	60	57	4 0– 8 0	4 0 8 0
10	1 25	32	68	4 0– 8 0	4 0 8 0
11	1 4	100	62	10 0–20 0	10 0 –20 0

isolated rabbit uterus The precision, however, depends to a great extent upon the method of calculation From the available data (Vos 1943, FOSTER & STEWART 1948) it can be concluded that the method presented here is at least as accurate and precise as Vos' method and 15–40 times more sensitive Moreover, the calculation involved is simpler, especially when the heights of contractions are themselves taken as response

The possibility of utilizing the method in pharmaceutical control analysis presents itself the method might be of value also in pharma cological work Thus LANZ, CERLETTI & ROTHLIN (1955) employed the antagonism to serotonin on rat uterus to investigate how LSD is distri buted in organism The oxytocic effect of ergometrine, though not suitable for quantitative work (BERDE & STURMER 1962, SUND 1963), could then be of some value as criterion of identity

Summary

Ergometrine can be determined by its anti serotonin action on isolated rat uterus when magnesium ions (usually 1 mM) are maintained in the bath In symmetrical (2 + 2) assays 11 solutions unknown to the operator were assayed The uterus was stimulated by 30–100 ng serotonin/ml every 6 minute and inhibited by 1 4–20 ng ergometrine/ml (usually 2–8 ng), added 2 minutes before the stimulant The mean departure from theoretical was −1 7% (range −9 6 to +8 6), the harmonic mean of ' index of precision" 0 060, the range of fiducial limits (6 groups of doses) 85–117% to 92–108%

Ergometrinine and *lumi*-ergometrine behaved qualitatively like ergometrine but were only about 2% and less than 1% as active, respectively Methylergometrine, being a stronger antagonist than ergometrine, and ergotamine could be differentiated from ergometrine on the basis of their behaviour

Acknowledgements

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Methylergometrine and ergotamine The next homologue, methylergometrine, and the main ergot alkaloid, ergotamine, were qualitatively distinguishable from ergometrine When a submaximal dose of ergometrine was repeatedly given, the inhibition usually increased slightly from the first to the second administration and then remained constant As already stated, the "after-effect" was negligible On giving methylergometrine or ergotamine, the inhibition continued to increase through several administrations (fig 3), also the effect persisted for a longer time With ergotamine the inhibition could indeed continue to *increase* long time after the antagonist had been washed out of the bath

The dissimilarities make it difficult to compare the three compounds quantitatively A procedure for doing this should take into account both intensity and duration of inhibition No doubt, however, methylergometrine is a stronger antagonist than ergometrine Taking the greater molecular weight and the time factor into consideration, ergotamine is also fairly active

Methylergometrine and ergotamine cannot be determined by the present method unless it is modified by using recovery doses of serotonin

Difficulties For this method 30 uteri from different rats have so far been employed Of these only 2 were discarded One showed pronounced tachyphylaxis to serotonin, 1 mM magnesium chloride was unsufficient to completely abolish the oxytocic effect of ergometrine on the other When this happened once later (assay no 11) the concentration was through 2 mM raised to 6 mM

A higher magnesium concentration should also be tried if the uterus tends to discriminate between different doses of antagonist rather in its latency period than in its height of contraction

If originally causing a large effect, the dose of serotonin chosen may become too large when, as occasionally, sensitivity increases during assay

Discussion

Many drugs have more or less selective anti serotonin action on isolated rat uterus Therefore the method described here is no more absolutely specific than already existing ones For the analysis of simple solutions of ergometrine and its main decomposition products it has however proved useful Particularly since existing methods have been subjected to criticism it is held to be a valuable supplement

The most reliable of existing *in vitro* methods appears to be that of Vos (1943), which is based upon the latency period for oxytocic effect on

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From The Department of Pharmacology, University of Copenhagen
(Professor Knud O Møller M D)

The Composition of Oedema Fluid Provoked in Mice by Oestradiol.

By

Eigill Hvidberg, Laszlo Szporny and Hans Langgaard

(Received October 14 1963)

In a series of publications from this laboratory the effect of oestradiol monobenzoate treatment on the connective tissue of mice of the Leo strain has been used in the study of connective tissue ground substance (SCHMIDT 1958, HVIDBERG & SCHOU 1959, HVIDBERG & JENSEN 1959, HVIDBERG 1959, HVIDBERG & SCHMIDT 1959, JORGENSEN & SCHMIDT 1962, HVIDBERG, JENSEN HOLM & LANGGÅRD 1963, LANGGÅRD JENSEN HOLM & HVIDBERG 1963) Tables 1 and 2 summarise the results of these investigations Prominent findings are hydration of the tissue and quantitative and qualitative alterations in the hyaluronic acid, a characteristic constituent of the ground substance For *in vivo* studies of connective tissue, treatment with oestradiol in these animals thus produces a useful ground substance ' model

A precise determination of quantitative changes in the various components of connective tissue is, however, rendered difficult by lack of a stable basis of calculation The commonly used principle of calculating on basis of 100 g of fat free solids is correct only on the assumption that the weight of fat free solids is not altered by the treatment SZPORNY, LANGGARD & HVIDBERG (1964) recently reported a method for quantitatively studying the composition of acute inflammatory oedema and based their calculations on the total contents of the components in skin samples marked out symmetrically on the skin surface The inflammatory reaction was confined to one of the areas, the other side serving as a control In our study we have tried to modify this principle of calculation for the oedema fluid provoked by oestradiol

In contrast with localised experimentally induced acute oedema, the

REFERENCES

Barger, G . Pharmacology of ergometrine (and ergometrinine) p 177–194 in *Hdb exper Pharmacol* Erg w Bd 6, Springer, Berlin 1938

Beauvillain, A.. Titrage des préparations post-hypophysaires par leur seuil d'action sur l'uterus isolé de rat adulte *Compt rend Soc Biol* 1943, **137**, 284–285

de Beer, E J & P. E Tullar The biological estimation of ergometrine *J Pharmacol exper Ther.* 1941, **71**, 256–260

[illegible] the presence of [illegible], for pharmacological assays on isolated preparations *Brit J Pharmacol* 1954, **9**, 24–30

Foster, G E. & G A Stewart The stability of ergometrine preparations, chemical and biological studies *Quart J Pharm Pharmacol* 1948, **21**, 211–218

Gaddum, J H : Bioassays and mathematics *Pharmacol Rev* 1953, **5**, 87–134

Gridgeman, N T Mathematics of biological assay. *Nature* 1944, **153**, 461–462

Hellberg, H On the photo-transformation of ergot alkaloids *Acta Chem Scand* 1957, **11**, 219–229

Jensen, K Briseid & A M Vennerød Determination of adrenaline on the isolated serotonin-stimulated rat uterus *Acta pharmacol et toxicol* 1961, **18**, 80–88

Lanz, U , A Cerletti & E Rothlin Über die Verteilung des Lysergsäure diäthylamids im Organismus *Helv Physiol Pharmacol Acta* 1955, **13**, 207–216

Pennefather, J N The estimation of ergometrine on the rat uterus *J Pharm Pharmacol* 1961, **13**, 60–61

Rothlin, E Beitrag zur differenzierenden Analyse der Mutterkornalkaloide *Schweiz Med Wchnschr* 1938, **19**, 971–974

Schild, H O A method of conducting a biological assay on a preparation giving repeated graded responses illustrated by the estimation of histamine *J Physiol* 1942, **101**, 115–130

Schild, H O pA, a new scale for the measurement of drug antagonism *Brit J Pharmacol* 1947, **2**, 189 206

Stoll, A & W Schlientz Über Belichtungsprodukte von Mutterkornalkaloiden *Helv Chim Acta* 1955, **38**, 585–594

Sund, R B On the actions of ergometrine on isolated rat uterus in absence and presence of magnesium ions *Medd Norsk Farm Selskap* 1963 To be published

Swanson, E E , C C Hargreaves & K K Chen The question of assaying ergotocin, the new ergot principle *J Am Pharm Assoc Sci Ed* 1935, **24**, 835–839

Vos, B J Use of the latent period in the assay of ergonovine on the isolated rabbit uterus *J Am Pharm Assoc Sci Ed* 1943, **32**, 138 141

Votava, Z & I Podvalova Pharmacological studies of ergometrine and methylergometrine *Acta pharmacol et toxicol* 1957, **13**, 309–318

White, A C A comparison of some of the ergot alkaloids *Quart J Pharm Pharmacol* 1944, **17**, 95 102

Wick, H J & C E Powell The effect of estron and stilbestrol on the response of rabbit s uteri to ergonovine *J Am Pharm Assoc Sci Ed* 1942, **31**, 46–47

Table 1.

Effect of treating mice of the Leo strain with oestradiol monobenzoate for one week

Connective tissue constituents in mg or meq /100 g fat free solids	fat water hexosamine hydroxyproline sodium chloride potassium	decreases about 25% increases 70–100% increases 90–100% decreases about 20 % increases about 200% increases about 120% increases about 20%
Blood	haemoglobin concentration pH plasma standard bicarbonate plasma sodium plasma chloride plasma potassium	no alterations

Table 2.

Further properties of skin from mice treated with oestradiol monobenzoate

molecular weight of isolated acid mucopolysaccharides		increased about 40%
spreading reaction		inhibited
absorption rate	paw oedema	normal
	from the back (urethane)	delayed
tensile strength of linear wounds		normal
histochemical colour reactions	metachromatic staining staining with Alcian blue staining with Hale s blue	greatly increased
	staining after hyaluronidase	no staining
	PAS staining	no staining
dehydration causes		water to decrease more than hexosamine
treatment with cortisone causes		water to decrease more than hexosamine
mild degrees of acidosis cause		dehydration and sodium mobilisation
mild degrees of alkalosis cause		no alterations

more "chronic" hydration induced by treatment with oestradiol is generalised, and control samples could therefore not be obtained from the same animals

Alternatively a comparison was made between skin samples of equal surface areas from treated and untreated animals, which is an acceptable procedure provided the area in question is unaltered by the treatment and that preformed components do not disappear from the tissue during treatment

The purpose of this communication thus is to further knowledge about connective tissue ground substance by using a new method for calculating the quantitative changes in the specific ground substance model produced by oestradiol treatment Further, the acute inflammatory process has been studied in animals after treatment with oestradiol in order to throw some light on the role of the ground substance in the process of inflammation

Methods

White male mice of the Leo strain weighing 23–27 g were used The animals were maintained under constant environmental conditions with free access to water and a standard diet

Groups of animals were injected with oestradiol monobenzoate (Ovex ® Leo) 10 µg in 0 1 ml arachis oil subcutaneously 4 and 6 days before the experiments Other groups of animals served as controls

Acute inflammatory oedema was provoked in one out of two areas marked out symmetrically on the depilated skin of the anaesthetised animal by application of pure xylol for two minutes A complete description of the procedures has recently been sent to press (SZPORNY LANGGÅRD & HVIDBERG 1964)

Determinations of water fat hexosamine hydroxyproline sodium chloride and potassium were carried out as previously described by HVIDBERG JENSEN HOLM & LANGGÅRD (1963) and LANGGÅRD JENSEN HOLM & HVIDBERG (1963)

In order to determine how comparable are skinsamples of equal surface area before and after treatment with oestradiol six mice were depilated and marked in the usual way but only on one side of the back After one week of treatment the other area was marked out the animals were then killed and the two were samples excised and weighed

Plasma hexosamine concentrations were determined in five oestradiol treated and five untreated animals Blood was drawn from the inferior cava vein under light ether anaesthesia The analytical procedures were the same as for tissue hexosamine determinations

Results

In tables 1 and 2 previous results obtained by treating mice of the Leo strain with oestradiol are summarised

The values in table 3 demonstrate that a defined area of the skin is not altered during oestradiol treatment In six animals, the average difference in weight between one area marked out before treatment and a symmetrically situated area marked out after treatment did not exceed the experimental error

Table 4 shows the total contents of water, fat and fat free solids in skin samples of equal surface areas from oestradiol treated and control animals and in the oedema fluid It is to be noted that the oedema fluid contained approximately 8 per cent fat free solids and that the amount of fat showed an absolute decrease of approximately 25 per cent during treatment

The weights of inflammatory oedemas induced by identical stimuli in skin samples of equal surface areas from oestradiol treated and control mice are shown in table 5 The absolute figures are not significantly higher in the treated group, although the percentage weight increase is smaller

Table 6

Analytical results for *total* contents of hydroxyproline hexosamine sodium, chloride and potassium of skin samples of equal surface area from oestradiol treated mice with xylolinduced inflammatory changes of the skin from oestradiol treated mice without inflammation and from a group of control animals

	No	hydroxyproline (mg)	hexosamine (µg)	sodium µeq	chloride µeq	potassium µeq *mean ± e m*
Oestradiol treated + xylol treated	21	3 52	870	68 9	61 8	17 9 ± 0 89
Oestradiol treated	21	3 23	703	47 6	42 7	16 8 ± 1 09
Control	24	3 24	216	11 9	13 4	10 1 ± 0 44

Table 7

Composition of oedema provoked by oestradiol
The figures are derived from the average values of the oestradiol treated group and of the control group therefore no statistical computations have been made The different fat contents of the two groups have been taken into account

	fatfree sol ds •.	hydroxyproline mg 100 ml	hexosamine mg/100 ml	sodium (meq/l)	chloride (meq/l)	potassium (meq/l)
Concentration in oestrad ol provoked	7 7	0	180	133	109	24 8

Table 3.

Weights of corresponding skin samples from six mice One area was marked out on the depilated skin before treatment with oestradiol After a week of treatment with oestradiol and with the same frame, another area was marked out on the opposite side, and the skin pieces were excised according to the markings (see text)

Mouse no	Area marked out before oestradiol treatment	Weight of right side	Weight of left side	The weight difference as percentage of the smaller side
1	right	520 mg	515 mg	+1 0%
2	–	466 mg	478 mg	−2 5%
3	–	682 mg	682 mg	0 0%
4	left	692 mg	664 mg	−4 0%
5	–	488 mg	508 mg	+4 0%
6	–	545 mg	502 mg	−8 0%
				Average −1 5%

Table 4.

Total contents of water, fat and fat-free solids of equal skin samples from treated animals and control animals and of the provoked "oedema"

	Total weight mg	Water mg	fat mg	fat free solids mg
Oestradiol treated animals (n = 21)	516	406	40	70
Control animals (n = 24)	260	157	54	49
"Oedema"	256	249	−14	21

Table 5.

	n	Oedema	
		mg mean ± *s e m*	as percentage of original weight
Oestradiol treated animals	21	170 ± 17	34 9
Control animals	31	126 ± 10	50

In table 7 the composition of the oedema fluid provoked by oestradiol is shown Since the figures are simply differences between average values for two groups of animals, no statistical computations could be made The different fat contents of the two groups were, however, taken into account A hexosamine concentration of 180 mg/100 ml and a potassium concentration of 24 8 meq/l were noted

In table 8 the concentrations of fat free solids, hexosamine, sodium and chloride in acute inflammatory oedema from oestradiol treated and control mice are compared with the plasma concentrations of normal mice No statistically significant differences can be demonstrated

The figures in table 9 show that the concentration of hexosamine in plasma is unaffected by the treatment with oestradiol

Discussion

The study reported here refers to two widely different conditions of the connective tissue, both characterised however, by considerable hydration of the tissue they are acute inflammatory oedema and slowly developing oedema provoked by oestradiol

Calculations on the composition of the oedema fluids present tricky problems and need careful consideration

The usual principle of calculating on the basis of 100 g fat free solids does not take into consideration any possible increase in the amount of fat free solids That this may lead to incorrect conclusions is demonstrated by the figures in tables 4, 7 and 8, showing that both types of oedema in fact contain 8 per cent of fat free solids

If, on the other hand, the components of the oedema fluid provoked by oestradiol are calculated on the basis of absolute values, the criteria laid down in the introduction must be fulfilled The figures of table 3 show that the skin area excised for analyses is unaltered by treatment with oestradiol and that the absolute values of the untreated animals are therefore acceptable as control values for the absolute values of the treated animals The second requirement, however, that no *preformed* component should disappear from the skin during treatment, *seems to* be unrealised The decrease in fat content, at least, is highly significant *It should* be pointed out, however, that the contents of hydroxyproline are unchanged, which suggests that the tissue structure is in other respects *unaffected*

In conclusion it can be stated that both methods of calculation are inadequate, but that employed simultaneously *they* can yield complementary information

It has previously been shown (SZPORNY, LANGGÅRD & HVIDBERG

Table 8.

Concentrations of fat free solids, hexosamine, sodium and chloride in xylolprovoked acute inflammatory oedema of oestradiol treated mice and of a control group of mice, compared with the plasma concentrations from untreated mice

	n	fat free solids % *mean s e m*	hexosamine mg/100 ml *mean s e m*	sodium meq/l *mean s e m*	chloride meq/l *mean s e m*
Acute inflammatory oedema of oestradiol treated mice	21	7 3 ± 0 74	101 ± 17 6	142 ± 16 9	104 ± 12.8
Acute inflammatory oedema of ontrol mice	16	8 5 ± 0 51	99 ± 7 9	144 ± 5 0	110 ± 5 7
Plasma	23	8 2[1]	93[2]	134[3] ± 2 2	109[3] ± 1 0

[1] from the literature
[2] n = 5
[3] from a previous report (LANGGÅRD JENSEN HOLM & HVIDBERG, 1963)

Table 9

Concentrations of hexosamine in the plasmas of five mice treated with oestradiol monobenzoate and five controls *For comparison the contents of hexosamine and water in the skin are also given*

		plasma hexosamine (mg/100 g plasma)	tissue hexosamine (mg/100 g fat-free solids)	tissue water (g/100 g fat free solids)
oestradiol treated	1	104 0	1128	522
	2	93 7	1296	594
	3	86 2	1037	528
	4	79 9	968	485
	5	90 3	804	455
	average	90 8	1047	517
untreated	6	101 7	501	306
	7	99 1	432	288
	8	94 6	475	320
	9	86 6	475	294
	10	82 5	527	329
	average	92 9	482	307

Table 6 gives the analytical results for the total contents of hydroxyproline, hexosamine, sodium, chloride and potassium in skin samples of equal surface areas from oestradiol treated mice with inflammatory changes of the skin, from oestradiol treated mice without inflammation and from a control group

emphasise that the determination of tissue hexosamine gives a reliable index of acid mucopolysaccharides contents

This is, however, not true if the changes are caused by an acute filtration of plasma proteins, as for example in the acute inflammatory reaction

Investigations on experimentally induced inflammatory oedema in oestradiol treated mice might yield information about the role of the ground substance in the inflammatory reaction In spite of a pre-existing severe overhydration of the tissue, the inflammatory oedema is virtually greater than in normal mice (table 5) The composition of the oedema is indistinguisable from that of inflammatory oedema in untreated mice, i e the same as plasma (table 8)

Apparently the increased and probably fully utilised water binding capacity of the hyaluronic acid increases the absolute size of the inflammatory oedema It appears that the abnormal water status of the connective tissue has no influence on the inflammatory reaction Neither does the condition of the ground substance interfere with the composition of the inflammatory oedema

Summary

The composition of the oedema provoked by oestradiol in mice has been examined The calculations were based on the total amounts of the components in skin samples with identical surface areas from treated and control animals

The oedema fluid contained fat free solids, sodium and chloride at [illegible]

hyaluronate

During treatment with oestradiol the fat-contents of the skin decreased by 25 per cent

The plasma concentrations of hexosamine were not altered by the treatment

The quantitative and qualitative changes in the connective tissue ground substance provoked by oestradiol, and the concommitant overhydration of the tissue had no effect on the formation or composition of a superimposed acute inflammatory oedema

Acknowledgement

This investigation was carried out in "The Laboratory for Investigation [illegible] rant from the [illegible]

(1964) that acute inflammatory oedema has in fat-free solids, albumen, hydroxyproline, hexosamine, sodium, chloride and potassium the same composition as blood plasma and probably consists of a bulk filtrate of the plasma (see also table 8). As demonstrated by table 7, oedema fluid provoked by treatment with oestradiol contains fat-free solids, sodium and chloride at the same concentrations, whereas the concentrations of hexosamine and potassium differ from the plasma concentrations in a striking way. In spite of the fact that no increase in the amount of cells can be observed (JORGENSEN, personal communication), the potassium contents are increased by approximately 70% at the same time as the hexosamine contents are doubled (table 1). The hexosamine values can be taken as a correct measure of the synthesis of acid mucopolysaccharides in the tissue during oestradiol treatment. The increase observed can account for approximately 10% of the total increase in fat-free solids. The greater part of the remaining solids probably consists of ground substance proteins synthesised or accumulated in the tissue, since no change in collagen contents is recorded.

These findings emphasise the fact that the oedema provoked by oestradiol is essentially different from the passive enlargement of the extracellular space seen in acute inflammatory oedema. This is clear also from the fact that it is possible to produce an acute inflammatory oedema on top of the oedema provoked by oestradiol (see tables 5, 6 and 8).

In contrast with this, oedema fluid provoked by oestradiol resembles strikingly the oedema of the late inflammatory reaction (SZPORNY, LANGGÅRD & HVIDBERG (1964). Considering the totally different pathophysiological conditions, the similarities between the two types of oedema are remarkable. In the late phases of the inflammatory reaction the regeneration of tissue has probably already begun by production of acid mucopolysaccharides. The hyaluronic acid is presumably synthesised first, since the turn-over of this compound is rapid, approximately 48 hours, (SCHILLER *et al.* 1955).

The accumulation of potassium in both oestradiol treated animals and in the late inflammatory reaction can be explained in relation to the increased amount of hyaluronic acid. By in vitro experiments KULONEN (1952) showed that the K/Na ratio in the water phase attached to hyaluronic acid was 3-4 times higher than in plasma. The possibility therefore exists that hyaluronic acid, which at the existing pH is predominantly present as a salt, exists as potassium hyaluronate.

The values in table 9 show that treatment with oestradiol has no effect on the hexosamine concentration of plasma. This is not surprising, since plasma hexosamine is derived from substances (glucoproteins) other than the acid mucopolysaccharides of the connective tissue. Th[illegible]

Acta pharmacol et toxicol 1963, 20, 253–266

From the Research Laboratories of AB Astra, Sodertalje, Sweden

Effects of Various Compounds on Adrenergic Mechanisms Recorded by Changes in Heart Rates of Anaesthetised Mice

By

S B Ross

(Received October 17, 1963)

For the pharmacological testing of substances with a direct sympathomimetic activity or interfering with catecholamine mediated responses by some other mechanisms, a rapid *in vivo* method is of practical value The method to be described is based upon measurements of heart frequency in anaesthetised mice With minor modifications this test seems to be useful for determining sympathomimetic activity and adrenergic β-receptor blocking effect as well as of the inhibition of monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT) produced by various compounds

Methods

In vivo studies

Mice, anaesthetised with 60 mg/kg of pentobarbital sodium intraperitoneally, were placed on an electrically heated pad to maintain a constant body temperature The ECG's of three mice were recorded simultaneously on a Grass Model 5 Polygraph Two hook electrodes were placed subcutaneously in each foreleg and a clip (earth) was put on the tail The heart rate was calculated from the record and tabulated

The sympathomimetic effect was studied after intravenous administration of the test substance An ECG was first recorded for 10 sec periods three times with five minute intervals before the injection One minute after the injection the recordings were continued at five minute intervals for one hour unless the heart rate returned to the control value before this time

The adrenergic β-receptor blocking effect of a substance was studied by comparing the stimulating effect on the heart rate of intravenously injected isoproterenol sulphate (10 μg/kg) in untreated mice and in animals pre treated with the substance given intraperitoneally 20 min before the isoproterenol The ECG was recorded

REFERENCES

Hvidberg, Eigill Effects of dehydration and fasting on the water binding mechanism of connective tissue *Acta pharmacol et toxicol* 1959, 16 28–37

Hvidberg Eigill & C E Jensen Changes in the molecular weight of acid mucopolysaccharides in connective tissue due to hormone treatment dehydration and age *Acta chem Scand* 1959, 13, 2047–2056

Hvidberg, E, J Jensen Holm & H Langgård Sodium, potassium and chloride in connective tissue *Acta pharmacol et toxicol* 1963, 20, 131–139

Hvidberg Eigill & Arne Schmidt Effect of prolonged cortisone treatment on subcutaneous connective tissue in mice *Acta pharmacol et toxicol* 1959, 15, 293–299

Hvidberg Eigill & Jens Schou The effect of oestradiol monobenzoate on connective tissue, especially on subcutaneous absorption *Acta pharmacol et toxicol* 1959, 15, 207–216

Jørgensen, O & A Schmidt Influence of sex hormones on granulation tissue formation and on healing linear wounds *Acta chir Scand* 1962, 124 1–10

Kulonen, E On the relation of hyaluronic acid to the water and electrolyte metabolism *Acta physiol Scand* 1952, 27, 82–90

Langgård, H, E Hvidberg & L Szporny The chemical composition of the acute inflammatory oedema throughout its formation 1964, to be published

Langgård, H, J Jensen Holm & E Hvidberg Connective tissue electrolytes during acidosis and alkalosis in normal and oestradiol treated mice *Acta pharmacol et toxicol* 1963, 20 121–130

Schiller, S, M B Mathews, L Goldfaber, I Ludowieg & A Dorfman The metabolism of mucopolysaccharides in animals Studies in skin utilising labeled acetate *J Biol Chem* 1955, 212 531–535

Schmidt, Arne The influence of cortisone and oestradiol on the amounts of hexosamine and water in skin *Acta pharmacol et toxicol* 1958, 14, 350–358

Szporny, L, H Langgård & E Hvidberg *Quantitative investigations on the composition of acute inflammatory oedema* To be published 1964

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three times before injection of the test substances and also at five minute intervals before and up to one hour after the injection of isoproterenol

COMT inhibition in vivo was determined in the same manner as the adrenergic β receptor blockade i e the substance to be tested was injected intraperitoneally and 20 minutes later a challenging dose of 10 μg/kg isoproterenol was administered intravenously

MAO inhibition capacity in vivo was tested in the same manner as the COMT inhibition except that tyramine hydrochloride 1 mg/kg intravenously was used instead of isoproterenol

To facilitate comparisons the doses of the heart stimulating agents given alone were selected so as to produce an increase in heart rate of about 150–200 beats per minute The duration of tachycardia was defined as the time from the injection of the sympathomimetic amine to the moment when the increase in heart rate had fallen to half its maximum value

In vitro studies

Catechol O methyl transferase (COMT) activity COMT activity *in vitro* was determined according to AXELROD ALBERS & CLEMENTE (1959) with minor modifications Mouse brain was homogenized in one volume of isotonic KCl solution with a MSE homogenizer An aliquot of the supernatant fraction of the centrifuged homogenate (14000 × g for 20 min) was incubated with 2 8 mμmol DL noradrenaline 7 ^3H (260 mμc) 2 μmole $MgCl_2$ 20 mμmol cysteinhydrochloride 80 mμmole S adenosylmethionine 28 μmole phosphate buffer at pH 7 8 and 37 C for 90 min and with the inhibitor in at least three different concentrations The final volume was 0 5 ml The normetanephrine formed was extracted after the addition of 1 ml of 0 5 M borate buffer and 3 g of solid NaCl with 15 ml of a mixture of toluene and isoamyl alcohol (3 2) The radioactivity in 5 ml of the organic phase was determined by liquid scintillation counting after addition of 1 ml ethanol and 10 ml of 0 4% 3 4 diphenyloxazole and 0 01% β bis [2 (phenyloxazolyl)] benzene in toluene The COMT inhibiting effect of the various substances was compared to that of pyrogallol at a 50 per cent inhibition level Pyrogallol like the other substances tested was used in at least three concentrations and run in parallel with the test substance at each determination

The substrate activity of isoproterenol for COMT compared to that of adrenaline was tested according to AXELROD & TOMCHICK (1958) The 3 methoxy products were measured in a Zeiss spectrophotofluorometer by excitation at 285 mμ and reading the fluoroscence at 315 mμ (uncorrected values)

The noradrenaline contents of the mouse hearts were determined by the method of SHORE & OHLIN (1958) Six hearts were pooled for each determination

Results

1 *General observations*

Several substances that induce tachycardia in other species were studied for their ability to increase heart rate in mice All injections intravenously Atropine, which in some species increases hea

no effect Nicotine, 1 mg/kg and DMPP (1, 1 dimethyl-4-phenylpipera zinium iodide), 1 mg/kg, were ineffective also in atropinised mice Caffeine was also without effect at 5 mg/kg Guanethidine and bretylium increased the heart rate at a dose level of 10 mg/kg This action may be related to a noradrenaline releasing effect Adrenergic α receptor blocking com pounds, such as dibenamine, phentolamine and chlorpromazine, had no effect on isoproterenol induced tachycardia

2 *Effects of some sympathomimetic amines*

In many species isoproterenol is known to increase the heart rate (KONZETT 1940) In this respect it was the most potent of the compounds included in our study (fig 1 A and table 1) The duration of the tachycardia, as defined above, induced by 10 µg/kg isoproterenol sulphate was about 10 min

Adrenaline and noradrenaline also increased heart rate, but to produce the same effect approximately two and four times the isoproterenol dose, respectively, had to be given (fig 1 B and 1 C) In comparison with isoproterenol the activities of 3, 4-dihydroxy norephedrine and dopamine were only $\frac{1}{8}$ and $\frac{1}{100}$, respectively The results are summarised in table 1 Of the other sympathomimetic amines tested, those meta

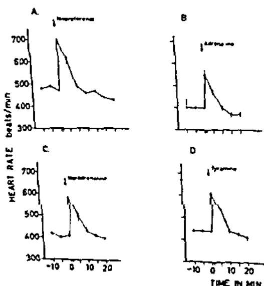


Fig 1 The tachycardiac action of some sympathomimetic amines on anaesthetised mice. A Isoproterenol sulphate 10 µg/kg i v B Adrenaline bitartrate 20 µg/kg i v C Nor adrenaline bitartrate 40 µg/kg i v D Tyramine hydrochloride 1 mg/kg i.v

Table 1.

Increase in heart-rate (Δ HR) produced by some sympathomimetic amines injected i v into mice anaesthetised with pentobarbital Heart-rate figures represent the increases above control levels

Amines	Dose mg/kg	Number of animals	+Δ HR beats/min	Duration in min
dl-Isoproterenol sulphate	0 010	9	210	10
	0 0025	3	140	5
l-Adrenaline bitartrate	0 040	3	195	16
	0 020	11	166	9
l-Noradrenaline bitartrate	0 040	9	187	8
	0 020	3	120	3
Dopamine hydrochloride	1 0	3	210	4
l-3, 4-Dihydroxy-norephedrine	0 050	3	180	4
dl-3-Hydroxy-norephedrine	0 100	3	170	8
l-Phenylephrine	1 0	3	135	26
dl-Ethyl-phenylephrine (Effortil ®)	0 1	3	120	5
l Synephrine	1 0	3	150	6
Tyramine hydrochloride	5 0	6	260	14
	1 0	3	180	6
Hordenine sulphate	20	3	140	6
dl-Ephedrine hydrochloride	2 0	3	260	>60
	1 0	3	270	>60
dl-Amphetamine sulphate	0 5	3	170	17
	5 0	3	290	>40

Table 2.

Effect of pre-treatment with reserpine and α-methyldopa on tyramine induced tachycardia in anaesthetised mice Reserpine (5 mg/kg) and α methyldopa (400 mg/kg) were injected i p 20 hours before the tyramine hydrochloride (20 mg/kg i v) Heart-rate figures represent the increases above control levels (Δ HR)

Pre-treatment	Number of animals	Δ HR beats/min (± *s e m*)
–	15	234 ± 17
Reserpine	8	79 ± 13
α Methyldopa	8	263 ± 18

hydroxylated were found to be more active than the unsubstituted and those para-substituted (table 1) Fig 1 D demonstrates the effect of tyramine, which is short-lasting, in contrast to that of *e g*, ephedrine or amphetamine

Pre-treatment of the animals with reserpine, 5 mg/kg intraperitoneally 20 hours before the experiment, inhibited the tyramine-induced tachycardia (table 2) This observation is in agreement with results found in

Table 3

Noradrenaline-releasing effect of reserpine and α methyldopa on the hearts of mice *in vivo* Reserpine (5 mg/kg) and α methyldopa (400 mg/kg) were given 20 hours before the mice were killed

Pre treatment	Number of determinations	Heart noradrenaline content as percentage of normal
Reserpine	3	8
α Methyldopa	3	11

other species (CARLSSON *et al* 1957) α-Methyldopa, on the other hand, did not inhibit the tyramine response when 400 mg/kg were given intraperitoneally 20 hours before tyramine The two substances cause almost complete disappearance of catecholamines from the heart 20 hours after injection (table 3), which is the same as the effects on other species (BERTLER, CARLSSON & ROSENGREN 1956, HESS *et al* 1961)

3 *Adrenergic β receptor blockade*

Dichloroisoproterenol (DCI) is known to block the effects of sympathomimetic amines on adrenergic β-receptors (POWELL & SLATER 1958) of the type generally considered to be involved in the tachycardia pro-

Table 4

Effects of adrenergic β-receptor blocking substances given i p on isoproterenol induced tachycardia in mice Isoproterenol sulphate (10 μg/kg) was injected i v 20 minutes after the blocking substances Heart rate figures represent increases above control levels (Δ HR)

Agent	Dose mg/kg i p	Number of animals	Δ HR after blocking substance beats/min	Δ HR after isoproterenol beats/min
		9	–	210
Dichloro-isoproterenol	0.25	3	150	110
	1.0	3	230	70
hydrochloride	5.0	3	330	30
Dimethyl-dideshydroxy	0.25	3	10	160
isoproterenol	1.0	3	170	80
hydrochloride (H 13 57)	5.0	3	160	40
Nethalide hydrochloride	1.0	3	75	135
	5.0	5	150	140
	10.0	3	100	80
	20.0	7	100	100
Rutin	100	3	100	70
Quercetin	100	3	225	120

Table 5

Sympathomimetic effects of some adrenergic β receptor blocking substances Heart rate figures represent increases above control levels (Δ HR)

Agent	Dose mg/kg i v	Number of animals	Δ HR beats/min
Dichlorisoproterenol hydrochloride	0 05	3	150
	0 25	6	215
	0 50	3	260
Dimethyldideshydroxy isoproterenol hydrochloride (H 13/57)	0 05	3	150
	0 25	3	2?0
	0 50	3	340
Nethalide hydrochloride	2	3	110
	4	2	120
	8	2	150

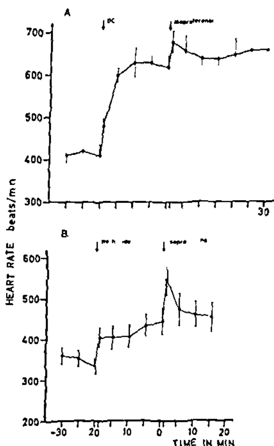


Fig 2 The tachycardiac effect and the action on isoproterenol induced tachycardia in mice by two adrenergic β receptor blockers injected 20 min before isoproterenol sulphate 10 μg/kg i v A Dichloroisoproterenol hydrochloride 1 mg/kg i p B Nethalide hydrochloride 20 mg/kg i p

duced by, e g, isoproterenol Fig 2 A and table 4 show that the tachycardia induced by isoproterenol was considerably decreased by DCI 0 25–5 0 mg/kg intraperitoneally At these doses DCI in itself gave a considerable increase of heart rate which may at least partly mask the effect of isoproterenol DCI induced tachycardia even at 0 05 mg/kg i v (table 5) These observations of the effect of DCI in mice are the same as those found in other species (MORAN & PERKINS 1958, DRESEL 1960)

The recently described compound nethalide (BLACK & STEPHENSON 1962) had evidently less sympathomimetic effect in itself than DCI judged by its effect on heart rate in mice However, inhibition by this compound of the adrenergic β receptors is also less marked (fig 2 B and table 4 and 5) Dimethyldideshydroxyisoproterenol (H 13/57) was closely similar to DCI in sympathomimetic effect and adrenergic β receptor inhibition

Among other compounds tested some flavonoids such as rutin and quercetin in large doses were found to counteract the isoproterenol induced tachycardia probably by adrenergic β receptor blockade (table 4)

4 *Catechol O methyl transferase (COMT) inhibition*

Pyrogallol is known to be a COMT inhibitor (AXELROD & LAROCHE 1959) and potentiates the actions of catecholamines (BACQ 1936) Isoproterenol has been found to be more readily affected by pyrogallol in mice than the naturally occurring catecholamines (ROSS 1963) It has no effects

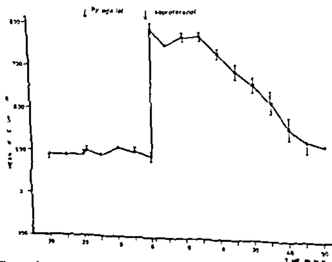


Fig 3 The prolonging action of pyrogallol 100 mg/kg i p on isoproterenol induced tachycardia in mice Pyrogallol was injected 20 min before isoproterenol sulphate 10 µg/kg i v

on adrenergic α receptors, which may indirectly influence heart rate and was therefore chosen as the *in vivo* substrate for COMT The action of 100 mg/kg i p pyrogallol on isoproterenol induced tachycardia is demonstrated in fig 3

The properties of isoproterenol as a substrate for COMT were submitted to some *in vitro* experiments, in which the product formed by incubating isoproterenol with liver extract from mice in the presence of S adenosylmethionine could be extracted under the same conditions as metanephrine The product, probably metaisoproterenol had a fluorescence spectrum resembling that of metanephrine The excitation maximum of metanephrine is at 285 mμ and that of the isoproterenol product at 279 mμ The fluorescence maximum of both compounds is found between 310 and 320 mμ If the specific fluorescence of the isoproterenol product is assumed to be equal to that of metanephrine 0 05 and 0 07 μmol of metanephrine and metaisoproterenol respectively, were found under the conditions used Isoproterenol may be a slightly better substrate for COMT than adrenaline

Besides pyrogallol, some other known inhibitors of COMT were tested Of these compounds 4 methyltropolone and 4 isopropyltropolone were the most potent in prolonging the tachycardia induced by isoproterenol This result is in agreement with that of BELLEAU & BURBA (1961) who have reported that 4 methyltropolone in the rat has about 30 times the potency of pyrogallol as a COMT inhibitor In our own study it was found to be about 10 times as potent as pyrogallol in mice (table 6)

Table 6

– … COMT

Substances	Dose mg/kg i p	Number of animals	Δ HR beats/min	Duration in min	COMT inhibition in vitro
–		9	210	10	
Pyrogallol	200	3	240	60	1
	100	3	200	33	
	50	3	210	15	
d Catechin	80	3	190	34	3
Quercetin	50	3	220	13	0 2
Catechol	40	3	240	18	0 1
4 Isopropyltropolone	25	3	250	39	1
	10	3	270	20	
4 Methyltropolone	20	3	240	37	1
	5	3	180	36	

The *in vitro* inhibition potencies of these tropolones, on the other hand, were about equal to that of pyrogallol under the conditions used

d Catechin had higher COMT inhibitory action than pyrogallol *in vitro*, about equal to that of pyrogallol *in vivo* Because of its β-adrenergic blocking effects quercetin could not be tested in larger doses than 50 mg/kg intraperitoneally, in which dose it was only slightly active as a COMT inhibitor *in vivo*, *In vitro* quercetin is much less potent than pyrogallol Catechol *in vitro* had only one tenth of the activity of pyrogallol as a COMT inhibitor Its COMT inhibitory effect at 40 mg/kg intraperitoneally was also slight At this dose catechol produces toxic symptoms in mice This is at variance with WYLIE, ARCHER & ARNOLD (1960) who reported that catechol augmented the pressor response of adrenaline in cats

5 *Monoamine oxidase (MAO) inhibition*

Pheniprazine (Catron ®), a known MAO inhibitor (HORITA 1958), at a dose of 5 mg/kg *intraperitoneally* was first used to study the effect on

Table 7

The effect of MAO inhibition on tachycardia induced by sympathomimetic amines in mice Pheniprazine was injected 3 hours before the amines Heart rate figures represent increases above control levels (Δ HR)

Amines	Dose mg/kg i p	Number of animals	Pheniprazine mg/kg i p	Δ HR beats/min	Duration in min
dl Isoproterenol	0 010	3	5	210	13
sulphate	0 010	9	0	210	10
l Adrenaline	0 020	6	5	150	6
bitartrate	0 020	11	0	166	9
l Noradrenaline	0 040	5	5	135	7
bitartrate	0 040	9	0	187	8
Dopamine	1 0	3	5	310	39
hydrochloride	1 0	3	0	210	4
l 3 4 Dihydroxy	0 050	3	5	180	6
norephedrine	0 050	3	0	180	4
dl 3 Hydroxy	0 100	3	5	170	>60
norephedrine	0 100	3	0	190	8
l Phenylephrine	1 0	3	5	190	>60
	1 0	3	0	130	26
dl Ethyl phenylephrine	0 25	3	5	170	18
(Effortil R)	0 25	3	0	160	20
l Synephrine	1 0	3	5	180	40
	1 0	3	0	150	6
Tyramine	1 0	3	5	250	43
hydrochloride	1 0	3	0	180	6
dl Ephedrine	1 0	3	5	270	>60
hydrochloride	1 0	3	0	270	>60
Hordenine sulphate	20	3	5	280	19
		3	0	140	6

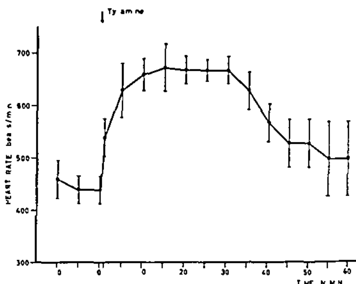


Fig 4 The prolonging action of pheniprazine hydrochloride 5 mg/kg i p on tyramine induced tachycardia in mice Pheniprazine was injected 3 hours before tyramine hydro chloride 1 mg/kg i v

the duration of a tachycardia that had been produced by some sympa thomimetic amines (table 7) Isoproterenol, adrenaline and noradrenaline showed no prolonged actions The effect of dopamine, on the other hand, was markedly prolonged after pheniprazine These results are in agreement with the observation by BLASCHKO (1952) that dopamine is a better substrate for MAO than epinephrine or norepinephrine

Among the other sympathomimetic amines, those with an unsubstituted side chain were most sensitive to MAO inhibition Fig 4 demonstrates the effect on tyramine-induced tachycardia Compounds that are N methylated, such as phenylephrine and synephrine, also seemed to be sensitive to a blockade of the MAO enzyme On the other hand, methyl substitution at the β carbon in the side chain seems to eliminate the sensitivity to the enzyme Such amines as amphetamine and ephedrine had generally a long lasting effect (table 1) However methardrine (3 hydroxynorephedrine), which belongs to this group of sympatho mimetic amines was potentiated by pheniprazine, although this sub stance is not deaminated by MAO according to the general rule that phenylisopropyl derivatives are not attacked by this enzyme (BLASCHKO 1952) The results are summarized in table 7

In another series of experiments tyramine, 1 mg/kg intraperitoneally, was used as substrate for MAO *in vivo* and the potencies of some MAO inhibitors were evaluated Table 8 demonstrates that tranylcypromine and pheniprazine were the most potent of the compounds investigated Next in order were nialamide and iproniazide This order of activity is

Table 8

Prolongation effects of some MAO inhibitors on tyramine induced tachycardia in mice. The MAO inhibitors were injected i.p. 3 hours before tyramine hydrochloride (1 mg/kg i.v.). Methylene blue was given i.p. 20 minutes before tyramine. Heart rate figures represent increases above control levels (Δ HR)

MAO inhibitor	Dose mg/kg	Number of animals	Δ HR beats/min	Duration in min
	–	3	180	6
Iproniazide	50	3	270	33
	25	3	270	14
Nialamide	10	3	330	>60
	5	3	300	34
Phenelzine	5	3	250	43
	2.5	3	230	18
	1.25	3	180	6
Tranylcypromine	2.5	3	345	>56
	1.0	3	290	7
Methylene blue	50	3	330	19

the same as that found for other species by other methods (Horita 1958, Rowe *et al* 1959, Green & Erickson 1962). Tranylcypromine was found to possess a marked sympathomimetic effect of its own, and this effect was stronger than that of any other MAO inhibitor tested.

Discussion

The number of animals used in this investigation was small, and direct quantitative comparisons between different compounds can therefore only be made with some caution. However, the results have shown that heart rate recording in anaesthetised mice might be a useful method for testing substances that interact with adrenergic mechanisms. By this method sympathomimetic, adrenergic β-receptor blockade, COMT- and MAO inhibitory effects and reserpine like action may be evaluated. However the heart rate of anaesthetised mice seems to be less sensitive to certain indirectly acting substances, for example nicotine and DMPP, which in other species induce tachycardia. The reason for these differences has not itself been studied.

The difference between the effects of reserpine and α methyldopa on tyramine induced tachycardia does not seem to have been reported previously. Both substances release catecholamines from mouse heart to about the same degree, but only reserpine antagonized the effect of tyramine in mice. Potter, Axelrod & Kopin (1962) have shown the quantity of catecholamines in the heart that may be released by tyramine

to be only a small part of the total content of catecholamines Although α-methyldopa and reserpine in mice release catecholamines to a similar extent, it may still be that the small tyramine-sensitive pool of catecholamines may not be affected by α-methyldopa Reserpine, on the other hand, would then be assumed to release this pool of catecholamines in heart as well Another explanation of the tyramine-effect in the α methyl dopa-treated mice may be that α-methyldopamine or α-methylnoradrenaline formed from α-methyldopa is taken up by heart tissues and liberated by tyramine (CARLSSON & LINDQVIST 1962)

A comparison between the *in vitro* and *in vivo* effects of the COMT inhibitors included in this study shows that quantitatively the two methods sometimes show poor agreement This may in part be due to a difference in the way in which the compounds inhibit the enzyme The tropolone derivatives seem to inhibit non-competitively (BELLEAU & BURBA 1961) Pyrogallol is at least partly a non-competitive inhibitor (CROUT 1961) but pyrogallol also seems itself to be methylated by the enzyme (ARCHER *et al* 1960) Thus at larger substrate concentrations than those used in the present *in vitro* study, the tropolones will probably show greater COMT inhibition than pyrogallol, as previously reported by BELLEAU & BURBA (1961)

The prolonging effect of pheniprazine on the duration of tachycardia induced by sympathomimetic amines seems to be due to at least two different mechanisms The amines that are deaminated by MAO may be partly or entirely potentiated through the MAO inhibitory action of pheniprazine For potentiating the effect of methardrine, which according to the general rule about phenylisopropylamine derivatives is not deaminated by MAO (BLASCHKO 1952), some other action of pheniprazine would seem to be involved The course of methardrine inactivation *in vivo* in mice is not known An enzyme other than MAO may be responsible for the metabolism of methardrine, and this enzyme may be blocked by pheniprazine Another possible explanation is that binding to some cellular constituent is the first step in the inactivation of methardrine The pheniprazine-induced prolongation of methardrine-induced tachycardia may accordingly be caused by an interference of pheniprazine with this binding of methardrine Pheniprazine may also to some extent affect, by the same mechanism, the amines deaminated by MAO

Summary

A method based on heart-rate analysis on anaesthetised mice seems to be useful for pharmacological testing of compounds that interfere with sympathomimetic responses From the results obtained with differ-

ent model substances, certain conclusions may be drawn about (1) sympathomimetic effect, if a substance increases heart rate directly, (2) adrenergic β-receptor blockade, if a compound antagonizes the stimulating effect upon the heart produced by isoproterenol, (3) COMT inhibition if a substance prolongs the action of isoproterenol, and (4) MAO inhibition if a compound augments the action of tyramine Reserpine counteracted tyramine induced tachycardia in mice probably through its catecholamine releasing action α Methyldopa, however, which also considerably decreases the concentration of catecholamines in heart did not affect this action of tyramine

Acknowledgements

For generous supplies of dimethyldideshydroxyisoproterenol (H 13/57), nethalide and 4-methyltropolone I thank Dr H Corrodi, AB Hässle, Göteborg, Sweden For technical assistance I am indebted to Mrs Öie Haljasmaa and Miss Laila Gustafsson, AB Astra, Södertälje, Sweden

REFERENCES

[illegible] 5 68 72

Axelrod J & M J Laroche Inhibitor of O methylation of epinephrine [illegible]

Blaschko H Amine oxidase and amine metabolism *Pharmacol Rev* 1952 4 415–458

Carlsson A & M Lindqvist *In vivo* decarboxylation of α methyldopa and α methylmetatyrosine *Acta physiol scand* 1962 54 87 94

Carlsson A E Rosengren Å Bertler & J Nilsson Effect of reserpine on the metabolism of catecholamines *Psychotropic Drugs* Amsterdam 1957 363 372

Crout J R Inhibition of catechol O methyl transferase by pyrogallol in the rat *Biochem Pharmacol* 1961 6 47–50

Dresel P E. Blockade of some cardiac actions of adrenaline by dichloroisoproterenol *Canad J Biochem* 1960 38 375 381

Green H & R W Erickson Further studies with tranylcypromine (monoamine oxidase inhibitor) and its interaction with reserpine in rat brain *Arch intern pharmacodyn* 1962, 135 407–425

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REFERENCES

Archer S, A Arnold R K Kullning & D W Wylie The enzymic meth

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E

Bertler Å A Carlsson & E. Rosengren Release by reserpine of catecholamines from rabbits hearts *Naturwiss* 1956 43 521

Black J W & J S Stephenson Pharmacology of a new adrenergic beta receptor blocking compound (Nethalide) *Lancet* 1962 II (7251) 311 314

Blaschko H Amine oxidase and amine metabolism *Pharmacol Rev* 1952 4 415–458

Carlsson A & M Lindqvist *In vivo* decarboxylation of α methyldopa and

Crout J R Inhibition of catechol O methyl transferase by pyrogallol in the rat *Biochem Pharmacol* 1961 6 47–50

Dresel P E. Blockade of some cardiac actions of adrenaline by dichloro isoproterenol *Canad J Biochem* 1960 38 375–381

Green H & R W Erickson Further studies with tranylcypromine (mono amine oxidase inhibitor) and its interaction with reserpine in rat brain *Arch intern pharmacodyn* 1962, 135 407–425

Hess, S M, P H Connamacher, M Ozaki & S Udenfriend The effect of α methyldopa and α methyl meta tyrosine on the metabolism of norepinephrine and serotonin in vivo *J Pharmacol exp Ther* 1961, *134* 129–134

Horita, A Beta phenylisopropylhydrazine, a potent and long acting mono amine oxidase inhibitor *J Pharmacol exp Ther* 1958, 122, 176–181

Konzett, H Zur Pharmakologie neuer adrenalin verwandter Körper *Arch exp Path Pharmakol* 1940, 197, 41–56

Moran, N C [illegible] heart by [illegible] ride, the [illegible] 1958, 122, [illegible]

Potter, L T, J Axelrod & I J Kopin Differential binding and release of norepinephrine and tachyphylaxis *Biochem Pharmacol* 1962 11, 254–256

Powell, C E & I H Slater Blocking of inhibitory adrenergic receptors by a dichloro analog of isoproterenol *J Pharmacol exp Ther* 1958, 122 480–488

Ross, S B In vivo inactivation of catecholamines in mice *Acta pharmacol toxicol* 1963, 20, 267–273

Rowe, R P, B M Bloom S Y Pan & K F Finger Pharmacological studies with nialamide, a new antidepressant agent *Proc Soc exp Biol Med* 1959, 101, 832–836

Shore P A & J S Olin Identification and chemical assay of norepinephrine in brain and other tissues *J Pharmacol exp Ther* 1958 122 295–300

Wylie, D W, S Archer & A Arnold Augmentation of pharmacological properties of catecholamines by O methyl transferase inhibitors *J Pharmacol exp Ther* 1960, 130 239–244

Acta pharmacol et toxicol 1963, 20, 267–273

From the Research Laboratories of AB Astra Sodertälje, Sweden

In Vivo Inactivation of Catecholamines in Mice

By

S B Ross

(Received October 17 1963)

Several pathways for the biological inactivation of catecholamines *in vivo* have been suggested The most important enzymes are monoamine oxidase (MAO) and catechol O methyl transferase (COMT) AXELROD *et al* (1958) have shown that the methylating enzyme in the liver plays the major role in the inactivation of circulating and exogenously supplied catecholamines Less is known about the inactivation mechanisms at peripheral sympathetic nerve endings and in brain WHITBY, AXELROD & WEIL-MALHERBE (1961) have suggested that the first step in the inactivation is some sort of binding of the amines to tissue constituents and the second step enzymatic O methylation Other investigators (CROUT 1961, IVERSEN & WHITBY 1962) have reported results that also seem to support this type of inactivation

In the study reported here some results were obtained to suggest that in mice the binding of isoproterenol by tissues is considerably less than is that of the naturally occurring catecholamines

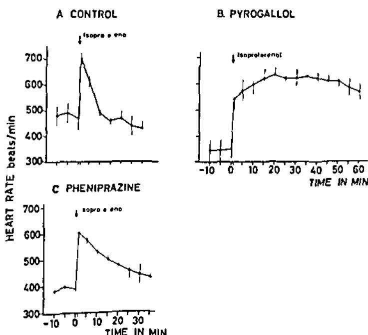
Methods

Tachycardic eff [illegible]

[illegible] catecholamine dose was selected so as to produce an increase in heart rate of about 150–200 beats per minute in animals not pre-treated

Influence of catecholamines on the uptake of tritiated noradrenaline in vitro The uptake of DL-noradrenaline-7-^{3}H by heart tissues *in vitro* was measured by the method of DENGLER, SPIEGEL & TITUS (1961) About 80 mg of heart (ventricle) tissue slices from mice were incubated in 2 ml of Krebs solution for one hour at 37°C with 0 02 µg/ml DL-noradrenaline-7-^{3}H hydrochloride, and the effect of different concentrations of the catecholamines on the uptake of radioactive noradrenaline was studied

The substrate specificity of isoproterenol to MAO The substrate specificity of isoproterenol to monoamine oxidase, compared to those of noradrenaline and adrenaline, was determined *in vitro* by incubating 6 µmol of the amines with a homogenate of mouse liver (300 mg) in 0 2 M phosphate buffer, pH 7 4, with and without 1 µmol pheniprazine (Catron ®) The final volume was 3 ml, and the incubation time was 60 minutes at 37°C The amines were extracted by mechanical shaking in an atmosphere of nitrogen with 15 ml of n butanol in the presence of solid sodium chloride The amines in 10 ml of the butanol phase were extracted by 3 ml of 0 01 N HCl, and the catecholamines were determined at pH 6 by the method of EULER & HAMBERG (1949) The decrease in catecholamine concentration after incubating without pheniprazine was compared with that obtained in presence of pheniprazine The difference was taken as an index of monoamine oxidase activity in the liver extract The substrate activities shown by the amines to monoamine oxidase were expressed as µmol deaminated amine per g tissue per hour



tachycardia produced by 10 µg/kg i v
Isoproterenol alone B Pyrogallol 200
nol C Pheniprazine hydrochloride, 5
before isoproterenol

Results

Increase of heart rate in vivo

Intravenous injection of a catecholamine into mice induces an increase in heart rate of short duration (Ross 1963) Figures 1 A, B and C show that the duration of tachycardia induced by isoproterenol was considerably prolonged by pyrogallol (a COMT inhibitor), but was much less affected by pheniprazine (a MAO inhibitor) The duration of the tachycardia induced by adrenaline was much less prolonged by pyrogallol than that of isoproterenol and was also little influenced by pheniprazine (fig 2 A, B and C) The combined pre treatment of mice with pheniprazine and pyrogallol clearly prolonged the tachycardia induced by adrenaline (fig 2 D) b t the d

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25 mg/kg i p , besides the other compounds, 10 minutes before the adrenaline injections The tachycardiac effect of noradrenaline was similar to that of adrenaline, and there was a relatively small increase in duration

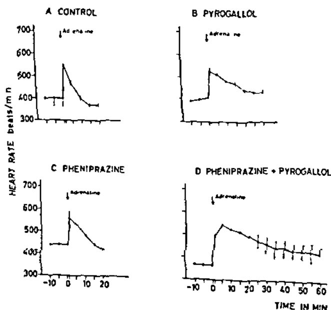


Fig 2. Action of pyrogallol and pheniprazine on tachycardia produced by 20 µg/kg intravenous *adrenaline* bitartrate in mice A Adrenaline alone. B Pyrogallol 200 mg/kg intraperitoneally 20 min before adrenaline C. Pheniprazine hydrochloride 5 mg/kg intraperitoneally 3 hours before adrenaline D Combined pre treatment with pheniprazine hydrochloride 5 mg/kg intraperitoneally 3 hours and pyrogallol, 200 mg/kg intraperitoneally 20 min before adrenaline

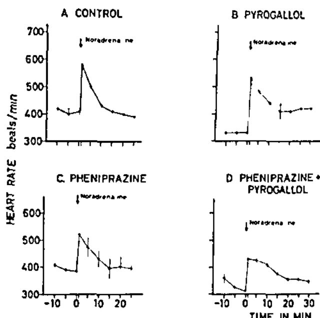


Fig 3 Action of pyrogallol and pheniprazine on tachycardia produced by 40 μg/kg intravenous noradrenaline bitartrate in mice A Noradrenaline alone B Pyrogallol ... -ine hydrochloride ... ned pre-treatment ... nd pyrogallol 200

when the mice had been pre-treated with pyrogallol or pheniprazine (fig 3 A, B and C) The effect of pyrogallol in prolonging the tachycardia induced by isoproterenol was much more pronounced than that of combined treatment with pheniprazine and pyrogallol on the noradrenaline-induced tachycardia (fig 3 D)

Effect of catecholamines on uptake of noradrenaline-7-^{3}H by heart tissues in vitro

The inhibition of catecholamines on the uptake of tritiated noradrenaline by heart tissue slices was taken as an index of the degree of uptake of these amines by the same mechanism Table 1 shows that isoproterenol affected the uptake of tritiated noradrenaline much less than either adrenaline or noradrenaline

The substrate specificities of noradrenaline, adrenaline and isoproterenol to MAO in vitro

To obtain a direct comparison of the substrate activities toward liver monoamine-oxidase of the three catecholamines investigated, the amines were incubated *in vitro* with liver homogenate from mice Table 2 shows

Table 1

Effects of noradrenaline adrenaline and isoproterenol on the uptake of tritiated noradrenaline by mouse heart tissue slices *in vitro* Noradrenaline 7 ³H concentration 0 02 µg/ml Incubation was performed for one hour at 37°C The final volume was 2 ml

Catecholamine µg/ml added		Percentage block of noradrenaline ³H uptake means of two determinations
L Noradrenaline	0 02	36 (41 31)
L Noradrenaline	0 2	66 (69 63)
L-Adrenaline	0 02	46 (49 43)
L Adrenaline	0 2	72 (72 72)
DL Isoproterenol	0 2	19 (29 8)
DL Isoproterenol	2 0	21 (20 22)

Table 2

Substrate activities shown by noradrenaline adrenaline and isoproterenol to monoamine oxidase from mouse liver homogenate *in vitro* Incubation of 6 µmol of the amines with and without 1 µmol pheniprazine at pH 7 4 for one hour at 37 C The final volume was 3 ml

Catecholamine	Deaminated amine µmol per g per hour
Noradrenaline	3 3
Adrenaline	4 3
Isoproterenol	3 0

that the three catecholamines have about the same substrate activities for liver monoamine oxidase from mice The observation that noradrenaline and adrenaline are about equally susceptible to deamination by MAO is in agreement with the findings of BLASCHKO RICHTER & SCHLOSSMANN (1937)

DISCUSSION

The different extents to which COMT inhibition affects the tachycardiac activities of isoproterenol and the naturally occurring catecholamines, adrenaline and noradrenaline, suggest some differences in the inactivation pathways of these amines The differences observed do not seem fully explicable by the observation that isoproterenol *in vitro* appears a slightly better substrate for COMT than adrenaline (Ross 1963) Also, since all

three amines are attacked by the monoamine oxidase from mouse liver *in vitro* to about the same degree, this enzymatic inactivation pathway would not seem to be responsible for the large *in vivo* difference between the amines

Whitby, Axelrod & Weil-Malherbe (1961) have suggested another type of inactivation mechanism for adrenaline and noradrenaline, namely, binding to some cellular constituents Crout (1961), investigating the cardiovascular effects of adrenaline and noradrenaline, confirmed the significance of this inactivation pathway Iversen & Whitby (1962) have shown that noradrenaline is taken up and bound *in vivo* by mouse tissues faster than adrenaline This does not seem to apply when heart slices are used *in vitro* (table 1) Isoproterenol appears to be taken up by heart tissues to a considerably smaller extent than noradrenaline and adrenaline, but probably by the same mechanism This could explain the great differences in duration of the tachycardia produced by isoproterenol and by the other two catecholamines when COMT is inhibited If this interpretation is correct, the results would also seem to emphasise the great importance of binding as the first step in the inactivation of noradrenaline and adrenaline

The effect of pheniprazine along with pyrogallol on the tachycardia induced by adrenaline and noradrenaline may be caused by its MAO inhibition However, pheniprazine may affect the action of sympathomimetic amines also in other ways Pheniprazine potentiates the effect on heart rate of methardrine, an amine not deaminated by MAO, according to the general rule that phenylisopropylamine derivatives are not attacked by that enzyme (Ross 1963) Pheniprazine has been found to block the release of noradrenaline from peripheral sympathetic nerve endings (Axelrod, Hertting & Patrick 1961) Uptake of tritiated noradrenaline by tissue slices *in vitro* is also inhibited by pheniprazine (unpublished results) These observations indicate that pheniprazine may affect the actions of noradrenaline and adrenaline also by interfering with uptake and binding reactions that, besides the MAO inhibition may explain the results obtained here for the tachycardia in mice induced by adrenaline and noradrenaline

The relatively weak inhibitory action of isoproterenol on noradrenaline uptake by tissues has prompted an investigation of the inhibitory action of other sympathomimetic amines (Ross & Renyi 1964) Several amines that only stimulate β receptors proved to be poor inhibitors of the uptake of noradrenaline, whereas amines with α receptor stimulating properties were found to be about a hundred times more active The possibility that the α receptor response depends on a mechanism similar to that determining uptake is being investigated

Summary

The duration of the tachycardiac effect of isoproterenol in mice was considerably prolonged when the catechol O methyl transferase (COMT) was inhibited, but was much less affected by monoamine oxidase (MAO) inhibition The effects of adrenaline and noradrenaline were only slightly prolonged by either COMT inhibition or MAO inhibition alone When both enzymes were inhibited simultaneously, the durations of tachycardia induced by adrenaline and noradrenaline were still much less than that of tachycardia induced by isoproterenol when COMT alone was inhibited Isoproterenol did not block the uptake of tritiated noradrenaline by heart tissues *in vitro* at concentrations ten to a hundred times greater than effective concentrations of adrenaline and noradrena line The importance of uptake and binding of the naturally occurring catecholamines as the first inactivation reaction, and the differences in this respect between these amines and isoproterenol are discussed

Acknowledgements

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REFERENCES

Axelrod J [illegible]

Axel [illegible]

Blaschko H D R[illegible] [illegible]
and oth [illegible]

Crout J R [illegible]
monoar [illegible] responses to norepinephrine
Proc Soc Exp Biol Med 1961 108 482–484

Dengler H J H E. Spiegel & E. O Titus Uptake of tritium labeled norepinephrine in brain and other tissues of cat in vitro *Science* 1961 133 1072 1073

Euler U S v & U Hamberg Colorimetric estimation of no[illegible]
the [illegible]

Iver[illegible]

Ros[illegible]

Ross, [illegible] B & L. Renyi To be published

Whitby L. G J Axelrod & H Weil Malherbe The fate of H^3 norepinephrine in animals. *J Pharm exp Ther* 1961 132 193 201

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From the Department of Pharmacology and Pathology
B J College, Poona 1, India

Protection by Indigenous Drugs Against Hepatotoxic Effects of Carbon Tetrachloride – a Long Term Study

By

S. M Karandikar, G. V. Joglekar, G. K. Chitale and J H Balwani

(Received October 7 1963)

Drug therapy of liver cirrhosis has been mainly symptomatic and disappointing, since much is still obscure about its etiology Many agents (REES 1962, FIUME *et al* 1961) being selected show beneficial effects against hepatic damage induced by carbon tetrachloride

JOGLEKAR *et al* (1963) have observed encouraging protective effects of LIV-52, a proprietary medicine, against carbon tetrachloride toxicity in mice It is claimed to contain reputed indigenous hepatic stimulants and has been reported effective (SHETH *et al* 1960, PATRAO 1957, SULE *et al* 1956)

We have now studied the long term effects of LIV-52 therapy and promethazine (phenergan ®) against carbon tetrachloride toxicity

Materials and Methods

Part A

We used 40 albino female rats of weight range 150–180 g divided in four equal groups one group as a control Animals in the other three groups were given carbon tetrachloride 0 2 ml mixed with liquid paraffin 0 2 ml subcutaneously twice a week One group of the three was given no protective agent

The remaining groups received either promethazine elixir 0 7 ml (2 5 mg/rat) or LIV 52 Pediatric drops 0 5 ml (30 mg/rat) daily by intragastric tube

The animals were observed for twelve weeks When death occurred during this period the liver was examined both macroscopically and microscopically The livers were weighed and their volumes were measured by displacement method

All animals surviving 12 weeks were killed and examined as outlined

Part B

We used eight male rabbits of weight range 1 5-1 8 kg to study the effects of LIV 52 All rabbits were given carbon tetrachloride 1 ml mixed with 1 ml of liquid paraffin subcutaneously twice a week as long as they survived Six of these received 2 g of LIV 52 powder suspended in 30 ml of water daily by intragastric tube

Histological examinations were made of the livers

Results

Table 1 gives the figures for survival among rats

Figure 1 gives the mortality of rats and rabbits

Table 2 gives the weights and volumes of livers from rats

Table 1.

LIV 52 reduced the percentage mortality in rats due to carbon tetrachloride considerably more than promethazine did

	No of Animals living after 12 weeks	% Mortality
Blank Control	10	0%
Carbon tetrachloride control	2	80%
Carbon tetrachloride & promethazine	2	80%
Carbon tetrachloride & LIV 52	7	30%

Table 2.

LIV 52 did not significantly alter the weight and volume of liver Promethazine showed significant increase in volume without a corresponding increase in weight

	Weight in g	Volume in ml
Blank Control	6 39 ± 1 08	5 ± 0 67
Carbon tetrachloride control	7 ± 1 4 (P > 0 05)	5 9 ± 0 9 (P > 0 05)
Carbon tetrachloride & promethazine	8 45 ± 1 8 (P > 0 05)	7 75 ± 1 2 (P < 0 01)
Carbon tetrachloride & LIV 52	6 74 ± 0 9 (P > 0 05)	5 45 ± 0 55 (P > 0 05)

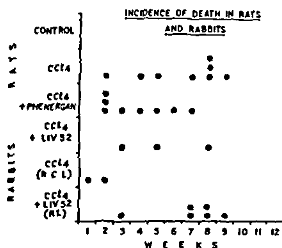


Fig 1 LIV 52 prolongs survival time in both rats and rabbits

1

2

3

4

Fig 2 (1–4) LIV 52 treated liver (4) appears like normal control liver (1) Carbon tetra chloride & promethazine treated livers (2 and 3) show gross cirrhotic changes

Table 3

Histological changes in rat & rabbit livers LIV 52 shows protective effects and marked regenerative activity the histological changes due to carbon tetrachloride being less severe

	Blank Control (10)	CCl_4 (7)	CCl_4 & promethazine (9)	CCl_4 & LIV 52 (10)	CCl_4 (Rabbits) (2)	CCl_4 & LIV 52 (Rabbits) (6)
Congestion	Nil	Moderate	Moderate	Nil	Moderate	Nil
Fatty change	Nil	Marked	Severe	Moderate	Severe	Moderate
Necrosis	Nil	Marked	Marked	Moderate	Marked	Moderate
Infiltration by chronic inflammatory cells	Nil	Moderate	Moderate	Moderate	Moderate	Moderate
Connective tissue (Reticulum stain)	Nil	*Thick bands	*Thick bands	*Thin streaks	Nil	Nil
Lobulation	Nil	Marked	Marked	Moderate	Nil	Nil
Regeneration	Nil	Moderate	Moderate	Marked	Nil	Moderate

Numbers in parenthesis indicate the number of livers of which histological examination was possible

* See photograph micro figs 8 9 & 10

Macroscopic appearance (fig 2) showed that in the carbon tetrachloride group (No 2) gross cirrhotic changes were noted in five animals and lobulation was obvious In the promethazine group cirrhotic changes were noticed in four animals, but there was only a suggestion of lobulation The livers of the LIV-52 appeared almost like control livers

Table 3 – Summarizes the results of the histological examinations

Comments

In a previous short term study on mice (JOGLEKAR *et al* 1963) it was observed that LIV-52 conferred a significant reduction in mortality and also appreciable protection against liver damage induced by carbon tetrachloride

This work was extended on a long term basis, and the period of study being increased to twelve weeks to discover whether or not LIV-52 has any effect on the cirrhosis induced by carbon tetrachloride. For the manifestation of cirrhotic changes a period of at least 8 weeks is necessary (WAHI 1956, FIUME 1961) Female rats were thus intentionally selected, since it was observed by us (JOGLEKAR *et al* 1963) that male mice succumbed quickly to the toxic effects of carbon tetrachloride GYORGI *et al* (1946) have noted a similar effect of sex on carbon tetrachloride toxicity

Though REES (1962) has reported the beneficial effects of promethazine against carbon tetrachloride, we were unable to confirm it in our earlier study Promethazine was included to study its effect in a long term trial

Even in this study we have observed the significant protection offered

by LIV-52 against carbon tetrachloride if the percentage mortality is taken as a criterion (table 1). Promethazine does not produce any difference in percentage mortality from that due to carbon tetrachloride alone Indeed, when if the incidence of death is examined (fig. 1) it seemes clear that promethazine has apparently potentiated carbon tetrachloride, since the deaths occurred quite early. Both in rats and rabbits LIV-52 prolonged survival time considerably.

There was no significant difference between the weights of the livers in any of the groups. However the promethazine group showed a significant increase in the volume of the liver (table 2). This may have been due to an increase in fat content of the liver, which could increase the volume without similarly altering the weight. Severe fatty degeneration observed histologically in the phenergan group (table 3) confirms this possibility.



Fig 3 Blank control

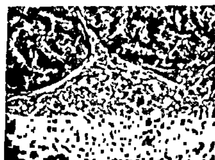


Fig 4 Carbontetrachloride control

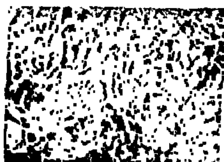


Fig 5 Carbon-tetrachloride plus promethazine

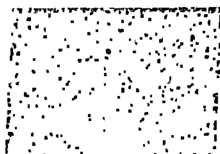


Fig 6 Carbon-tetrachloride plus LIV-52

Figs 3 to 6 The LIV-52 group shows remarkable protective effects histologically (Fig 6) compared with carbon-tetrachloride group (Fig 4) and promethazine group (Fig 5) Promethazine group shows excessive fatty infiltration

On histological examination (figs 3, 4, 5, 6) it was clear that the LIV-52 treated group showed considerably reduced fatty change and necrosis Hepatic structure was maintained in LIV-52 group Regenerative changes



Fig 7 Blank control



Fig 8 Carbon tetrachloride control



Fig 9 Carbon tetrachloride and promethazine



Fig 10 Carbon tetrachloride and LIV 52

Figs 7 to 10 (Reticulum stain) The LIV 52 group shows thin streaks of reticulum indicative of marked regenerative activity in the surrounding areas (Fig 10) Carbon tetrachloride and promethazine groups (Figs 8 and 9) show thick bands of reticulum and lobulation

were marked in this group compared with the others On reticulum staining (figs 7, 8, 9, 10), the thick bands of reticulum observed in the carbon tetrachloride and promethazine group were clearly absent in LIV-52 group, where we found thin compressed streaks of reticulum due to extensive regenerative changes in the surroundings Similarly, the lobulation so clearly seen both macroscopically and microscopically in the carbon tetrachloride and promethazine group was not observed in the LIV-52 group

The mechanism of this protective effect of LIV-52 remains to be elucidated, but we are impressed with its beneficial effect against the hepatotoxic action of carbon tetrachloride in prolonging the survival and changing the gross and the histological appearances of liver tissue This salutary effect is observed even in rabbits, the other species studied (fig 11 & 12)

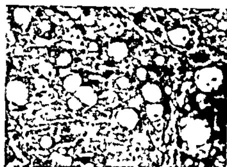


Fig 11 Carbon-tetrachloride control (rabbit)



Fig 12 Carbon tetrachloride plus LIV-52

Figs 11 & 12 LIV-52-treated rabbit livers show less necrosis and fatty change (Fig 12) compared with those four rabbits receiving carbon tetrachloride alone (Fig 11)

Summary

A protective effect of LIV-52 – an Indian indigenous proprietary medicine – is noticed against carbon tetrachloride poisoning in rats and rabbits, even in a long-term study.

Acknowledgements.

We thank Dr. F. J. Mendonca, Dean, B J. Medical College, for his encouragement and Shri. V. N. Joshi, College artist for photographic help.

LIV-52 was supplied by Himalaya Drug Co., Bombay and promethazine-phenergan ® by May & Baker, Bombay, India

REFERENCES

Fiume, L & G Favilli Inhibition of Experimental Cirrhosis by Carbon-tetrachloride following treatment with Aminoacetonitrile *Nature* (Lond) 1961, 189, 71–72

Gyorgi, P, J Seifter, R M Tomarelli & H Goldblatt Influence of dietary factors and Sex on the toxicity of carbon-tetrachloride in Rats *J Exp Med* 1946, 83, 449–462

Joglekar, G V, G K Chitale & J H Balwani Protection by Indigenous Drugs Against Hepatotoxic Effects of Carbon tetrachloride in Mice *Acta Pharmacol et toxicol* 1963, 20 73–79

Patrao, S Observations on LIV 52 *Journal of Indian Medical Profession* 1957, 4, 1878–79

Rees, K R Cellular injury by Drugs Symposium on Enzymes and Drug action – Ciba – foundation 1962 *J & A Churchill Ltd, London,* p 344–358

Sheth, S C, B J Northover, N S Tibrewala, U R Warekar & V S Karande Therapy of cirrhosis of liver and liver damage with indigenous drugs – experimental and clinical studies *Indian J Pediat* 1960 27, 204–211

Sule, C R, P M Sathe, M C Koshy & M S Deshpande Ascitis due to Liver deficiency treated with an indigenous drug *The Indian Practioner* 1956, 9, 357–362

Wahi, P N, H D Tandon & T P Bharadwaj Adrenal Cortex and hepatic cirrhosis *A M A Arch Path* 1956, 62, 200–214

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From The Department of Pharmacology University of Copenhagen
(Professor Knud O Møller M D)

Effect of Morphine on Tolerance to Nalorphine and its Metabolism

by

Torkell Jóhannesson

(*Received October 17 1963*)

Recently we have shown that rats made tolerant to morphine also showed an increased tolerance to the combined lethal action of morphine and nalorphine given simultaneously (JÓHANNESSON & MILTHERS 1963 b) In the same investigation it was also found that the concentration of nalorphine in the brain of the rats was higher when a dose of nalorphine was given together with morphine than if the same dose had been given alone These unexpected results suggested that morphine tolerant rats were also made tolerant to nalorphine and that morphine might interfere with the metabolism of nalorphine However this conclusion was based on experiments made with a different purpose from investigating the effect of morphine on the tolerance and metabolism of nalorphine Thus lethal doses of nalorphine had not been given alone to non tolerant rats and the results compared with those obtained on rats made tolerant to morphine For this reason we have thought it necessary to conduct experiments aiming directly at deciding whether or not morphine tolerant rats have been made tolerant to nalorphine also and whether or not the presence of morphine would also increase the amounts of nalorphine found in the brain of rats if the drugs were given together and the animals killed 30 minutes afterwards

Experiments and Methods

Adult male albino rats (140–250 g) were used Morphine tolerance in the rats was produced as described by JÓHANNESSON (1962 a) The tolerant rats were put on experiment 17–20 hours after the last daily injection of morphine Normal rats are referred to as non tolerant

Injection solutions and doses Morphine chloride was given in aqueous solutions containing 40 mg/ml, nalorphine chloride (Anarcon ®) was administered in aqueous solutions containing 30 mg/ml The drugs were given by intraperitoneal injections When both drugs were given to the same animals morphine was injected first and then nalorphine immediately afterwards from another syringe

Morphine was given in amounts of 320 mg/kg and nalorphine in amounts of 360 mg/kg or 150 mg/kg

The lower doses of nalorphine and the morphine dose were not lethal to the rats whether the drugs were given separately or simultaneously The higher doses of nalorphine (360 mg/kg) were not lethal to the tolerant rats, whereas the non tolerant rats usually died within 30 minutes after the injections Respiratory arrest was taken as a sign of death

The effect of morphine on nalorphine concentration in the brains of rats was studied In these experiments morphine, the lower dosage of nalorphine (150 mg/kg) or the mixture of morphine plus nalorphine was given to non-tolerant rats

Determination of morphine and nalorphine was performed polarographically, as described by JÓHANNESSON & MILTHERS (1963 a) In the investigation reported here, however, polarographic measurements were made in a greater volume (1200 µl, see below) than in the earlier ones, and larger electrolysis vessels (14 × 50 mm) accordingly were used By this technique the lowest measureable amounts are about 0 8–1 0 µg When small amounts of morphine plus nalorphine are added to brain homogenates of rats killed without any previous administration of drugs, the average recovery is about 95% for both morphine and nalorphine (cf also MILTHERS 1961) All results refer to µg ' free" morphine and nalorphine per g total wet brain

The analytical procedure includes paper chromatography, as described by BROSSI HAFLIGER & SCHNIDER (1955) The solvent mixture was amylene hydrate/di n buthyl ether/water in the ratio 80/7/13 Whatmann paper no 1, buffered with phosphate buffer to pH 6 3, was used Paper chromatography was continued for 16 hours at 22°C The R_F value of morphine was between 0 08 and 0 09 and that of nalorphine between 0 50 and 0 57

Morphine and nalorphine were eluted from the paper with N HCl according to the technique of CONSDEN, GORDON & MARTIN (1947) We eluted 400 µl during 30–60 minutes, 200 µl N-HCl were added to this volume and left over-night Then polarographic measurements were made after adding 200 µl M-KNO_2, 300 µl 20% (w/v) KOH and 100 µl 2% (w/v) methylcellulose (tylose) to give a total volume of 1200 µl (for details cf JÓHANNESSON & MILTHERS 1963 a)

Results

The lethal effect of nalorphine in morphine tolerant and non-tolerant rats

Twelve non-tolerant rats received 360 mg/kg nalorphine They died 14–110 minutes after the injections (average, 27 minutes) Convulsions were seen, but they did not invariably precede death in the rats Nalorphine was determined in the brain of six of these rats

Eight morphine tolerant rats were given 360 mg/kg nalorphine They all survived for at least 24 hours Further, for comparison four tolerant

Table 1

Nalorphine concentrations in the brains of morphine tolerant and non tolerant rats given 360 mg/kg nalorphine intraperitoneally

Morphine Tolerant Rats		Non Tolerant Rats	
Nalorphine in brain (μg/g)	Killed min after inj	Nalorphine in brain (μg/g)	Died min after inj
20 8	20	28 9	14
46 2	25	42 0	32
26 8	15	51 0	24
33 1	30	44 5	23
		31 7	16
		27 3	20
m = 31 7 μg/g	22.5 min	37 6 μg/g	21 5 min

rats received this dose of nalorphine and were killed at the same times as those of the spontaneous deaths of the non tolerant rats Their brains were removed for determination of nalorphine

The results of the nalorphine determinations are given in table 1 The nalorphine concentration appeared to be on an average higher in the brains of the non tolerant than in those of the morphine tolerant rats the difference, however, is not statistically significant (0 5 > P > 0 4)

The effect of morphine on nalorphine concentration in the brains of non tolerant rats given the drugs simultaneously

Three groups each of six rats, were given morphine, nalorphine (150 mg/kg) or morphine plus nalorphine The rats were killed 30 minutes

Table 2

The concentrations of morphine and nalorphine in the brains of non tolerant rats killed 30 minutes after intraperitoneal injections of 320 mg/kg morphine 150 mg/kg nalorphine or these quantities of morphine and nalorphine given simultaneously

Rats injected with morphine	Rats injected with nalorphine	Rats injected with morphine + nalorphine	
Morphine in brain (μg/g)	Nalorphine in brain (μg/g)	Morphine in brain (μg/g)	Nalorphine in brain (μg/g)
9 0	3 2	7 0	14 6
15 2	4 6	20 9	25 1
15 8	5 9	7 7	5 6
11 3	5 8	19 0	10 5
20 0	1 0	13 5	11 0
17 5	3 2	17 0	10 9
m = 14 8 μg/g	4 3 μg/g	14 2 μg/g	13 0 μg/g

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REFERENCES

Brossi, A, O Häfliger & O Schnider Oxymorphinane 6 Mitteilung Die papierchromatographische Bestimmung von Morphinderivaten und die Verfolgerung ihrer Ausscheidung beim Hund *Arzneimittelforschung* 1955, 5, 62–66

Consden R, A H Gordon & A J P Martin The identification of lower peptides in complex mixtures *Biochem J* 1947, 41, 590–596

Jóhannesson, T Morphine as an inhibitor of brain cholinesterases in morphine tolerant and non tolerant rats *Acta pharmacol et toxicol* 1962 a, 19, 23–25

Jóhannesson, T The concentrations of morphine in the brains of rats receiving morphine and neostigmine *Acta pharmacol et toxicol* 1962 b, 19, 286–292.

Jóhannesson, T & K. Milthers Determination of morphine *Radiometer News* 1963 a (in the press)

Jóhannesson, T & K. Milthers The lethal action of morphine and nalorphine given jointly to morphine tolerant and non tolerant rats. *Acta pharmacol et toxicol* 1963 b, 20, 80–89

Milthers K. Normorphine, nalorphine and morphine Quantitative separation and determination of small amounts in blood and tissues *Acta acol et toxicol 1961, 18, 199–206*

after the injections Determinations were made of morphine, nalorphine or both drugs in the brains The results are given in table 2 and show that nalorphine was found at *higher concentrations in the brains when* given with morphine than when given alone The difference is statistically significant ($0\,02 > P > 0\,01$) Morphine, on the other hand, was found at concentrations within the same range, whether given alone or with nalorphine

Discussion

If rats are injected daily with morphine in increasing amounts for weeks, they develop tolerance to its lethal effect (JÓHANNESSON 1962 a b) The results of the investigation reported here show that the morphine tolerant rats are also tolerant to the lethal action of nalorphine, as previously assumed by JÓHANNESSON & MILTHERS (1963 b) Further, *according to our own unpublished results, morphine tolerance in* the rats involves tolerance to the lethal effect of normorphine It seems therefore highly probable that the lethal effects of morphine, nalorphine and normorphine in the rat are due to the same mechanism

As metnioned above, the results of JÓHANNESSON & MILTHERS (1963 b) indicating that the concentration of nalorphine in the brain is increased when it is given together with morphine, were unexpected The new investigation confirms this observation It is thus substantiated that morphine can increase the amounts of nalorphine found in the brain of rats if given simultaneously in large doses by intraperitoneal injections However, elucidation of the mechanism of this phenomenon and of its possible significance in relation to the distribution of morphine and *related substances, must await the results of further experiments*

Summary

It is shown that morphine tolerant rats also are tolerant to the lethal effect of nalorphine

If morphine and nalorphine are given simultaneously by intraperitoneal injections to non tolerant rats, the brain concentration of nalorphine is found to be *significantly* higher 30 minutes after the injections than when measured after the injection of nalorphine alone The brain concentration of morphine is found to be within the same range, whether it has been given alone or with nalorphine

extracted from water into chloroform, whereas at a sufficiently high pH it will be soluble in water only and completely insoluble in chloroform

Another property, which is a consequence of the activated NH group, is the ability to form a resonating anion that absorbs ultraviolet light in alkaline solution The recorded spectrum of etosuximide shows little absorption at 240 mμ, but a strong maximum at 218 mμ when measured at pH 12 In acid solution its ultraviolet absorption is negligible These facts suggested the possibility of designing an analytical procedure based on the same principle as the well known method for determining barbituric acids, described by Lous (1950) The conditions have to be altered somewhat, as the substance is a much weaker acid than the barbituric acids and the molar extinction is much higher

Methods

The serum is extracted with chloroform At acid or neutral pH the partition coefficient between water and chloroform is 1 3 in favour of

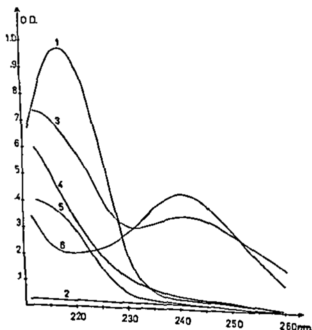


Fig 2 Spectral curves of (1) etosuximide at pH ~ 12 (2) the same at pH < 2, (3) 5 phenyl, 5-ethyl barbituric acid (phenemalum Ph Dan) at pH ~ 12, (4) the same at pH < 2, (5) 5-allyl, 5 isopropyl barbituric acid (allypropymalum Ph Dan) at pH < 2 (6) the same at pH ~ 12.

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From the Department of Biochemistry, University of Copenhagen, Denmark

Quantitative Determination of Etosuximide (Zarontin ®, α-methyl, - α-ethylsuccinimide) in Serum

By

Svend Erik Hansen

(Received October 29, 1963)

Etosuximide (= zarotin ® = α-methyl, α-ethylsuccinimide) was introduced a few years ago by Parke, Davis & Co under the provisional description PM 671 and has since then been used extensively and with considerable success in the treatment of epileptic disorders The reasons for wanting an analytical procedure for measuring this compound are several It has not been possible, for example, to determine whether accumulation occurs or not from its constant ingestion Neither has it been possible to follow diurnal variations in its concentration A contributory reason has been, that on one occasion from among a great number of successfully treated patients it seems to have been responsible for pancytopenia (KIORBOE et al 1964) Etosuximide is a colourless somewhat soft crystalline substance, with a melting point of about 40°C It is highly soluble in water, alcohol and ether and still more soluble in chloroform Chemically it is α-methyl, α-ethylsuccinimide, that is to say, it has some relation with the barbituric acids, as shown in fig 1

The NH-group is situated between two carbonyl groups in such a way that the hydrogen atom is activated, and the substance behaves like a weak acid This is important because under acid conditions it may be

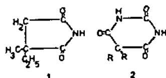


Fig 1 The formulae of etosuximide (1) and a 5, 5 disubstituted barbituric acid (2) show the relationship between them

A volume of 2 ml supernatant are transferred to a 1 cm quartz spectrophotometer cell and the absorbance is read against phosphate solution at 240 mμ and 218 mμ

Now 25 μl 6 N HCl are added to both reference and sample cell, and the readings are repeated

A blank determination is performed by carrying 0 5 ml of water through the same procedure as the serum

(0 5 ml of a serum, known not to contain etosuximide or barbiturates, may equally well be used for the blank determination)

Calculation

The extinction difference at 218 mμ (Δ_{218}) is corrected by subtracting the difference shown by the reagent blank, the concentration may be read from a standard curve, which needs only be prepared once for any one laboratory, being well reproducible

Any significant difference at 240 mμ indicates the presence of barbiturate, which must be identified by paper chromatography, e g by the

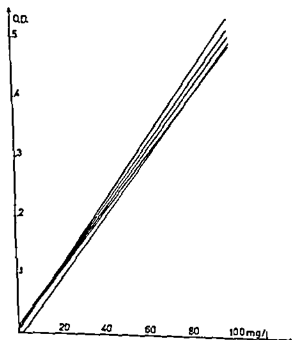


Fig 3 Consecutive standard curves (2 of the 6 practically identical and thus showing as one) constructed over 2 months, by adding varying amounts of etosuximide standard solutions to water or serum and subtracting the reagent blank

chloroform The extraction is therefore practically quantitative when 1 volume of serum is extracted with 20 volumes of chloroform

After filtering, the chloroform is re extracted with a solution of tri sodium phosphate, which has a pH value of 12 At any lower pH the extraction is not quantitative, a still more alkaline medium might damage the substance, although it shows relatively good stability in cold aqueous NaOH It is essential that the extractions be performed in glass stoppered tubes without lubricants, as *these are liable to interfere with the spectro* photometric measurements During our investigation Thunberg tubes have been used with satisfactory results The aqueous phase is centrifuged and the absorbance of the aqueous solution is measured at 240 and 218 mμ before and after acidification, this causes disappearance of that part of the absorbance at 218 mμ for which etosuximide is responsible, whereas there are no significant alterations at 240 mμ provided no barbituric acids are present The fall in absorption is directly proportional to the amount of etosuximide present

Reagents

Chloroform, Pharmacopea Danica or analytical grade Na_3PO_4 0 05 N (6 35 g Na_3PO_4, 12 H_2O dissolved in distilled water and made up to 1 liter)

HCl 6 N

Standard solutions of etosuximide 40 – 80 – 120 – 160 – 200 mg/100 ml distilled water

When 25 μl of each of these standard solutions are added to 0 5 ml of water and the analysis is performed as described below, the results will correspond to concentrations of 20 – 40 – 60 – 80 – 100 mg/litre respect ively

Procedure

In a glass stoppered test tube *0 5 ml of the serum* to be analysed and *10 ml of chloroform* are shaken vigorously by hand for about 3 min The stopper may be tightened with a drop of water if necessary but no lubricant should be used After being allowed to stand for a few minutes, the chloroform layer is filtered through ordinary filter paper into a clean test tube

4 ml of the extract and *5 ml of 0 05 N Na_3PO_4* are shaken in another glass stoppered tube for 3 min When the phases have separated, the aqueous phase is removed by pipette and centrifuged to spin down any suspended chloroform droplets 3000 r p m for a few min is sufficient

Acta pharmacol et toxicol 1963, 20, 291–294

From Home Office Forensic Science Laboratory,
Harrogate Great Britain

Cyanide Poisoning

By

A S Curry

(Received October 28 1963)

Cyanide deaths are usually straightforward, in that they involve suicides who ingest relatively large amounts of the sodium or potassium salts and die rapidly in obvious circumstances Analyses are rarely necessary, because of the characteristic smell of the stomach contents and the erosion of the mucosa, as a consequence there are few recorded concentrations of the poison in tissues The paper by HALSTRØM & MOLLER (1945) in which eight cases were described, describes the largest series and even there the level in the blood is only given for three

I have seen no results on deaths relating to hydrocyanic acid gas inhaled by humans, but two recent cases that came within my experience prompted me to compare them with seven deaths after oral ingestion In another case a man was accidentally splashed with molten sodium cyanide and died approximately ten hours later Consideration of these cases has produced results that may be of use forensic to toxicologists

Results

The method used has throughout been that of GETTLER & GOLDBAUM (1947) with the modification that the ferrous hydroxide test paper is prepared immediately before use CURRY (1963)

The results for the seven cases of oral I

Those of inhalation of HCN gas

The first of the inhalation cases accidental gassing in a chemical factory A blood

by the works doctor

method of WRIGHT (1954). From the spectrum of that particular barbiturate, its contribution to Δ_{218} can easily be calculated from the Δ_{240}. The Δ_{218} is corrected in this way, and the etosuximide concentration is read from the standard curve.

Results and Comments

Fig. 3 shows 6 successive standard curves prepared over two months by the method described. All points represent mean values of duplicates It will be seen that the slope shows virtually no change with time. Further, *recovery from serum is satisfactory, as the same standard curve is obtained* whether varying amounts of etosuximide are added to serum or to water. The extracts are stable for several hours After having been left overnight in the refrigerator before reading, a set of standards still gave correct results Critical factors are purity of reagents, cleanliness of glassware and an efficient spectrophotometer.

For this investigation a Unicam SP 500 spectrophotometer was used, but equipment such as Beckman DU and Zeiss SPQ II should also be satisfactory.

Summary

The antiepileptic drug etosuximide is briefly described and the reasons for needing an analytical procedure are given The procedure consists of extracting the drug from serum with chloroform, re-extracting it into aqueous trisodium phosphate solution and determining the absorbance of this solution before and after acidification with hydrochloric acid A method of correcting the presence of barbiturates is described, and results for standard curves are shown

Acknowledgements

A gift of pure crystalline zarontin ® from Parke, Davis & Co., Inc is gratefully acknowledged

The work described was begun at the laboratory of Kolonien Filadelfia, Dianalund and finished at the Biochemical Department of the University of Copenhagen.

REFERENCES

Kiørboe, E., J Paludan, E Trolle & E Overvad Zarontin ® (etosuximide) in the treatment of petit mal epilepsy and related disorders *Epilepsia* 1964, in press

Lous, P Quantitative determination of barbiturates *Acta pharmacol et toxicol* 1950, 6, 227–234

Wright, J T. A rapid method of identifying particular barbiturate derivatives in small samples of blood by paper chromatography *J Clin Path* 1954, 7, 61–65

Table 3

Victim splashed with molten sodium cyanide*

Source of blood	Time between death and post mortem examination	Time between death and analysis	Concentration in mg/100 ml
Femoral vein	14 hours	36 hours	nil
Carotid		36 hours	0 02
Carotid		44 hours	0 004

*) *The pathologist was Dr S Wray*

entered the building All soon felt dizzy, although they had weakness in the legs, they fortunately managed to reach the safety of fresh air, they required hospital attention I have been told by one of these officers that not one of them noticed the characteristic smell of hydrogen cyanide

The man who was splashed with molten sodium cyanide had the blood concentrations shown in table 3 A urine sample taken at post mortem showed a level of 0 05 mg/100 ml of cyanide, five times higher than the maximum found in urine samples taken from men after a period of work at the factory putting the solid cyanide into sacks

Discussion

The levels in the cases due to oral ingestion of relatively large amounts of cyanide are similar to those observed by HALSTRØM & MOLLER (1945) and GETTLER & BAINE (1938) In this series also all those who died were males

About the deaths from inhalation several points seem worth mention Analyses obviously must be performed as soon as possible, and the blood level may be as low as 0 05–0 1 mg/100 ml We have also found that putrefaction of blood, brain, liver and kidney samples can produce highly significant amounts of cyanide, this is another reason for determining this poison as soon as possible after receipt of the samples

It is also interesting that the lungs in the second inhalation case, in which the victim was exposed to a large concentration, showed a lower concentration than the blood, in the first case, when exposure was minimal, cyanide was not detectable in the liver or lungs Our failure to detect cyanide in the lungs on another occasion, where two suicides sprinkled sodium cyanide on trays inside a car and also took overdoses of sleeping capsules, should be of interest, although at the time we were using the silver nitrate titration method of cyanide estimation with

Table 1

Case No	Type of Poison	Time interval between ingestion and death	Amount in stomach contents
1	Very old sodium cyanide	found dead	2 mg
2	Sodium cyanide	5 minutes	100 mg
3	½ ounce potassium cyanide	30 minutes	100 mg
4	11 5% w/v aqueous potassium cyanide = 3 5 g	less than 25 minutes	164 mg
5	Potassium cyanide	found dead	108 mg
6	Sodium cyanide	found dead	230 mg
7	HCN solution, probably one ounce of 4%	found dead	20 mg

Table 1 continued

Concentrations in milligrams per 100 g

Case No	Blood	Liver	Brain	Pathologist
1	–	1 13	0 1	Dr W L Rose
2	1 1	0 6	0 2	Dr I S Stewart
3	0 25	0 25	0 25	Dr J Whitehead
4	1 0	1 0	0 3	Dr Gee
5	0 6	0 1	–	Dr Pinto
6	3 0	1 0	0 1	Dr I S Stewart
7	3 5	6 3	0 5	Dr Hainsworth

Table 2

Inhalation of HCN Gas

Case No	Time interval before death	Concentrations in milligrams per 100 g				Pathologist
		Blood	Liver	Brain	Lungs	
1	"a few minutes"	femoral 0 1 carotid 0 05	nil	0 01	nil	Dr S Wray
2	found dead	femoral 1 5	0 2	0 34	0 32	Dr Skeoch

ysis the next day As indicated in the table, blood samples from post-mortem examination analysed at the same time showed significantly lower values *In the second case the victim broke into a warehouse and* knocked the top off a canister of the gas intended for use as a ships' fumigant His mode of entry into the premises, by breaking a window, was discovered by a patrolling *police officer, and five officers subsequently*

Acta pharmacol et toxicol 1963, 20, 295–302

The Toxicity of the Rodenticide Castrix ® (2-chloro-4-diamethylamino-6-methylpyrimidine) and the Antidotal Effect of Vitamin B_6.

By

E. Knudsen

(Received October 29, 1963)

The search for new rodenticides during the last war led to the discovery of the rodenticidal action of 2-chloro-4-dimethylamino-6-methylpyrimidine, known as castrix ®*) (DuBois 1948). Its toxicity was studied by DuBois, Cochran & Thomson (1948), who found it to produce symptoms typical of central nervous system stimulants. Convulsions occurred after a latent period of 15 to 45 minutes, from either oral or intraperitoneal administration. The LD50 in the rat was 1.00 mg/kg by intraperitoneal and 1.25 mg/kg by oral administration. After intraperitoneal administration it was 0.42 mg/kg in mice, 2.66 mg/kg in guinea pigs, about 5 mg/kg in rabbits and about 0.5 mg/kg in dogs. Prolonged pentobarbital treatment was found to lower the toxicity to rats and dogs. Investigating a homicide committed by means of castrix ®-wheat, Goldbach (1950) concluded that the total lethal dose of pure castrix ® in man was about 50 mg. In studies on rats Lehman (1951) observed convulsions after a latent period of 45 minutes and found an approximate oral LD50 of 1.7 mg/kg. In a study on 22 rats Crampton & Voss (1952) administered drinking water contaminated with castrix ® for up to 50 days. Although the total 24 hours' uptake of castrix ® was 3–4 mg/kg, no pathological changes except a moderate depression of weight increase were noticed. Several observations, reviewed by Przygodda (1957), indicated that crop bearing birds are relative resistant to the commercial cereal bait

*) For reasons of brevity the trade name castrix ® was used throughout this paper. The formula is also written 2-chloro-4-methyl-6-dimethylaminopyrimidine.

a lower limit of detection of about 0 1-0 2 mg/100 ml The death after injection of HCN solution, quoted by POLSON & TATTERSALL (1959) is worthy of mention in this context because, apart from the tissue at the site of injection, the tissue levels were *not above those that can be obtained from 'normal'* tissue

Recovery after oral ingestion of cyanide must be rare, in one case of my own a wife took apart her husband s Monotheamin and Amytal capsules, emptied them and repacked them with 40% solid sodium cyanide The victim took one and ingested about 0 05 g of the poison He vomited and recovered completely

Summary

Toxicological analyses are reported for 2 fatal cases of hydrogen cyanide poisoning after inhalation, one in which the victim was splashed with molten sodium cyanide and seven cases of oral ingestion of cyanide

Acknowledgements

I am most grateful to the pathologists listed in tables 1, 2 and 3 for sending me the material from their cases and to H M Coroners for permission to publish I also wish to thank Dr J T B Bain for the urine samples from cyanide workers and Mr E R Rutter for assistance with the analyses

REFERENCES

Curry A S *Poison Detection in Human Organs* Charles C Thomas Springfield Illinois 1963 p 34

Gettler A O & J O Baine The toxicology of cyanides *Amer J Med Sci* 1938 195 182 198

Gettler A O & L Goldbaum Detection and estimation of microquantities of cyanide *Anal Chem* 1947 19 270–271

Halstrom F & K Møller The content of cyanide in human organs from cases of poisoning with cyanide taken by mouth With a contribution to the toxicology of cyanides *Acta Pharmacol et Tox col* 1945 1 18–28

Polson C J & R N Tattersall *Clinical Toxicology* English Universities Press London 1959

Table 1.

Acute toxicity of castrix ® to mouse rat, and chicks

Animal	Sex	Number of animals	Route of administration	LD 50 mg/kg
Mouse	female	60	oral	1.2
Mouse	both	96	s c	1.2
Mouse	female	42	i v	1.3
Rat	both	40	oral	1.5
Rat	both	60	s c	1.4
Chicks	both	40	oral	22.5
Chicks	both	24	i m	approx 20

Results

Toxicity of aqueous castrix ® solutions

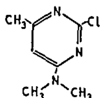
The toxicity of castrix ® was studied on mice, rats and chicks by administering aqueous solutions of the pure compound, the resulting LD50 values are recorded in table 1. The difference between the susceptibilities of chicks and other species is evident.

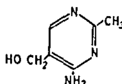
It was observed that the time interval from administration to onset of convulsions varied among individuals, but it was not related to the route of administration. The latency period was as long after intravenous as after oral administration. This interval appeared to be shorter in mice than in rats, and longer in chicks than in rats, but the individual variation was such as to make uncertain the statistical significance of this difference. The latency period did, however, shorten with rising dosage of castrix ® but was in no case observed to be shorter than 15 minutes.

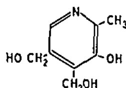
Toxicity of castrix ®-wheat

As the rodenticidal action of the commercial castrix ® wheat has been established for years, feeding experiments with rodents were undertaken on a few mice and rats only. One gram of castrix ®-wheat consisted of 18–23 grains and contained 1 mg of active substance. One single grain

divided into five groups and fed 20, 30, 40, 50, and 60 g of castrix ®- ... weight were
wheat per individual, corresponding approximately to levels of 14, 20
26 34 and 40 mg of castrix ® per ...
the castrix ®-wheat had previously
and was administered together wi... ...amount of







product containing 0.1 per cent of castrix ®, which was confirmed in experiments on the domestic fowl by GYLSTORFF (1962)

Because of the resemblance between the toxic symptoms due to and the molecular configurations of castrix ® and toxopyrimidine (fig. 1), the present series of experiments was begun; in preliminary tests vitamin B_6 proved to be a specific antidote in mice, lowering the toxicity of castrix ® more than hundredfold (KARLOG & KNUDSEN 1963)

Methods

In all, 335 albino mice, 334 albino rats and 105 chicks were used for this study.

The test substance castrix ® was 98% pure according to the manufacturer, our own investigations did not reveal impurities detectable by ultraviolet or infrared spectrophotometry or by gas chromatography The test substance was always administered as a sterile and approximately isotonic aqueous solutions in concentrations ranging from 0 2 to 10 mg/ml, so that excessive administration of fluid was avoided

Pyridoxine, pyridoxal and pyridoxal-5-phosphate of pharmaceutical purity were administered as sterile aqueous solutions with the pH raised to about 5 by addition of NaOH

Feeding experiments were carried out with the commercial cereal bait product Castrix ®-wheat (purple-dyed whole wheat containing 0 1% (w/w) of castrix ®) Rats and mice that had been deprived of food for six hours devoured the castrix ®-wheat voluntarily To chicks it was administered by forced feeding after 12 hours of food deprivation During the feeding experiments the animals always had free access to drinking water.

The toxicity tests were carried out by the method of KARBER (1931)

The experiments with *Saccharomyces carlsbergensis* were carried out by the method described in Pharmacopoea Nordica, vol IV, 1960, p 119–122

In each species the survivors were observed for five weeks A slight diarrhoea was always evident on the first day after the administration of castrix ®, but apart from that were no clinical signs differentiating the treated from the control animals Gross autopsy performed at the end of the observation period revealed no pathological changes In animals autopsied after acute death, only stasis of lungs, heart, kidneys, and gastrointestinal tract was observed

Antidotal effect of vitamin B_6

The antidotal effect of vitamin B_6 was previously observed in castrix ®-poisoned mice (KARLOG & KNUDSEN 1963), in which species simultaneous injections of 25 mg of vitamin B_6 raised the LD50 of Castrix to 137 mg/kg This antidotal action was reinvestigated in rats and chickens Quantities of 25 mg vitamin B_6 were injected simultaneously with up to ten times the LD50 of castrix for rats, and up to four times the LD50 for chicks, without occurrence of toxic manifestations in either species When the vitamin was administered after the beginning of convulsive symptoms (30–45 minutes after castrix ® administration), all toxic symptoms disappeared in about ten minutes Seizure prone animals would often go into convulsions when handled, and the optimal time for antidotal injections was found to be the period immediately after a seizure The effects of pyridoxine, pyridoxal, and pyridoxal 5 phosphate were found to be the same at the dosages chosen

As this dosage of 25 mg/kg had been fixed arbitrarily, pyridoxine doses of 1, 2, 5, 10, 20, and 25 mg/kg were tested on 144 rats that were simultaneously treated with castrix ® at doses of levels of 2, 5, 10, and 20 mg/kg and compared with control groups similarly treated with castrix ® but no pyridoxine Both compounds were administered subcutaneously.

Table 2

Surviving animals in 28 groups of 6 rats given simultaneous s c injections of castrix ® and pyridoxine in various doses

Pyridoxine dosage mg/kg	Animals in each group surviving Castrix doses of			
	2	5	10	20 mg/kg
0	1	0	0	0
1	4	4	3	2
2	6	6	6	2
5	6	6	5	5
10	6	6	6	6
20	6	6	6	6
25	6	6	6	6

water. In the other groups dry castrix ®-wheat was administered All chicks survived and only in the 40, 50, and 60 g groups were genuine seizures noticed, but sometimes repeated convulsions were observed for up to six hours. After complete disappearance of all toxic symptoms a large amount of grain was still palpable in the crop of each chick, including those in the group receiving soaked castrix ®-grain

Toxic symptoms.

Independently of the route of administration, the toxic symptoms were alike in the three species studied In chickens however, the toxic manifestations were more conspicuous, and this species showed the clearest departure from normal behaviour Ten to twenty minutes after administration ptosis and somnolence were evident, occasionally the legs suddenly gave way and the head drooped, but was not placed under the wing as in the normal sleeping position The next stage was one of arousal, accompanied by a persistent stereotyped food pecking from the empty cage floor If bits of wood or concrete, too big to swallow, were placed in the cage, the chickens would peck them and try to swallow them with the indefatigability of monomania In some cases the period of somnolence was interspersed with such food pecking episodes, but only exceptionally did chickens peck their cage-mates at this stage Given access to ground dry cereals, the chickens would start eating hungrily, even if their crops were distended by 60 grams of dry castrix ®-wheat Water was always at their disposal, but signs of abnormal thirst were never observed After a period of rising restlessness, the chickens would display an almost prototypical search and flight reaction, running sidewards to and fro along the cage bars, conveying to the observer the idea of a search for escape, attempting here and there to press their heads through the bars and suddenly, in an explosion of energy, pressing head, neck and wings through the fairly sturdy bars of the cage This phase would rapidly develop into a genuine seizure of convulsive running and flying movements Depending on dosage and individual vigor, the seizure would end fatally or leave the chicken stunned and prostrated in some unphysiological position until the next fit Chickens dying in convulsions had maximally extended wings and legs, distended thorax and extreme opisthotonus, so that the back of the head touched the saddle of the rump

In rodents the symptomatic picture was less clear on account of the normal restless activity of these animals, but it was possible to discern the same sequence of symptoms as in fowl The initial phase of ptosis and somnolence was clear, but not so the bulimy phase In rodents the biting frenzy was noticed immediately before and during the convulsive seizures, at which stage the animals would bite cage bars and odd objects, so that their lips and tongues bled profusely, as well as cagemates or even their own tails The search reaction was only evident in animals returned to their own cage after administration of the poison Otherwise it might be mistaken for the normal investigation of new surroundings, although the search phase was always accompanied by some spasticity of movement The flight reaction was sometimes indicated by perservering, heroic leaps towards the top of the cage, and convulsions began with enormously rapid jumps back and forth through the cage The seizure was accompanied by screaming and always left the animal with maximally

The toxic symptoms have been described in detail, as they in many respects reminded of reactions obtainable by electric stimulation of thalamic and hypothalamic structures (HESS 1948), *e g* ptosis, somnolence, bulimy, and search and flight reaction, suggesting that these symptoms might originate from identical areas In literature the only recorded case of castrix ® poisoning in man seems to be a homicide (GOLDBACH 1950) in which instance the toxic symptoms were only noticed by the lay murderess and reported to be somnolence, gastrointestinal pain, vomiting and diarrhoea During our investigation two medical cases of castrix ® ingestion by man have appeared, both of which were treated by gastric lavage and pyridoxine injections According to a personal communication by FROST, the only symptom noticed was somnolence, and in neither subject were toxic symptoms observed after treatment

Summary

The toxicity of 2 chloro-4-dimethylamino 6-methylpyrimidine (castrix ®) was investigated on mice, rats and chicks After oral administration the LD50 values were 1 2 mg/kg for mice, 1 5 mg/kg for rats, and 22 5 mg/kg for chicks Vitamin B_6 was found to be a specific antidote, injected in a dose of 25 mg/kg, it totally inhibited the toxic symptoms of castrix ® injected simultaneously at a dose level several times the LD50 The findings suggest that castrix ® in the animal organism may be metabolized to a vitamin B_6-antagonist

Acknowledgements

This study was aided by a grant from Statens Almindelige Videnskabsfond Special thanks are given to dr O Karlog, originator of this investigation, for liberal advice and help, to dr H Lieck and his staff (The Danish State Vitamin Laboratory) for donations of experimental animals and microbiological help and to A/S Agro-Kemi for generous supplies of pure castrix ® and castrix ® wheat

REFERENCES

Crampton J M & E Voss An investigation of the chronic toxicity and acceptability of Castrix *J Am Pharm Ass* 1952 41 135–138

DuBois K P New rodenticidal compounds *J Am Pharm Ass* 1948 37, 307 310

DuBois K. P K W Cochran & J F Thomson Rodenticidal action of 2-chloro-4-dimethylamino-6-methylpyrimidine (Castrix) *Proc Soc Exp Biol Med* 1948 67, 169 171

Goldbach H J Ueber eine tödliche Vergiftung mit dem Rattenvertilgungsmittel "Castrix" *Med Klin* 1950 45 961 963

Gylstorff J Die Giftigkeit von Castrix Giftgetreidekörnern für Hühner und Tauben *Bayer ... wirtsch Jb* 1962, 39 19 32.

The results are given in table 2, from which it is seen that 10 mg/kg of pyridoxine protected all animals against the lethal effects of up to 20 mg/kg of castrix ® This dose, however, did not fully inhibit the convulsions Reversible toxic symptoms were not taken into consideration, as exact criteria could not be decided upon, but protection against all toxic symptoms in rats was usually obtained by administering about twice as much pyridoxine as castrix ® In chicks, a species found to be less susceptible to castrix ®, the antidotal ratio of pyridoxine to castrix ® was somewhat higher

Microbiological investigation

On account of the evident antagonism between pyridoxine and castrix ® *in vivo*, the effect of castrix ® was studied on the vitamin B_6-dependent *Saccharomyces carlsbergensis* Castrix ® was added to the culture medium in concentrations ranging from one percent of the pyridoxine content up to 40,000 times the pyridoxine content of the medium In no instance did castrix depress the growth of the microorganism

Discussion

DuBois (1948) made the interesting observation that the toxicity of castrix ® to rats was about halved during the winter months It should therefore be noted that our experiments were performed in the spring and summer months They confirmed the previous findings, mentioned in the introduction, and it may be concluded that the toxicity of castrix ® is high for all mammal species examined and relatively low for crop bearing birds By oral administration of aqueous castrix ® solutions, the passage of which is not delayed in the crop, and by intramuscular administration of the poison to chicks it was, however, demonstrated that the existence of this organ is not solely responsible for the higher tolerance of crop bearing birds to castrix ® Otherwise, in the experimental feeding of castrix ®-wheat to chicks, the delay and storage function of the crop appeared to be the major reason for the complete lack of lethal effects of the cereal bait

The mechanism of action of castrix ® is still unknown, but the specific antidotal effect of vitamin B_6 and the toxic symptoms of castrix ® suggest that this compound is either a vitamin B_6-antagonist or is metabolized to such in the animal organism The failure to inhibit growth of *S carlsbergensis* could be interpreted as evidence for the latter hypothesis, which would also agree with the observed latency period between administration of the convulsant and the appearance of seizures, irrespective of route of administration

Acta pharmacol et toxicol 1963, 20, 303–308

From the Department of Pharmacology, Faculty of Medicine,
University of Gothenburg Sweden

Blockade of the Lactic Acid-Stimulating and Calorigenic Effects of Adrenaline or Isoprenaline by Chloroisoprenaline.

By

Lennart Lundholm and Nils Svedmyr

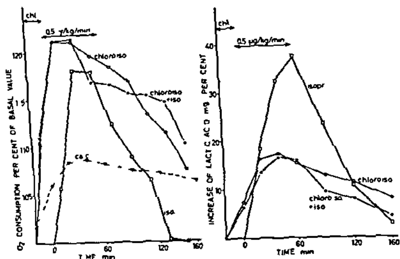
(Received November 4 1963)

A close relationship between the lactic acid stimulating and the calorigenic effects of adrenaline (LUNDHOLM 1949; LUNDHOLM & MOHME-LUNDHOLM 1960) and isoprenaline (LUNDHOLM & SVEDMYR 1964) has been demonstrated in experiments on rabbits It was therefore of interest to determine whether chloroisoprenaline blocked these effects, particularly as the latter drug, in experiments on the myocardium, had inhibited the phosphorylase activating effect of adrenaline (MAYER & MORAN 1960) and the glycogenolytic effect in rat skeletal muscle (KENNEDY & ELLIS 1963)

Method

The rabbits were prepared and their oxygen consumptions recorded as described in an earlier paper (LUNDHOLM 1949) The animals had been deprived of food for 16–18 hours before the experiment After recording the basal oxygen consumption for 60 minutes, an arterial blood sample was taken for determining the lactic acid content In one run of experiments chloro isoprenaline (Lilly) was then infused intravenously at a total dose of 15 mg/kg over a 10 minute period The drug was dissolved in 2 ml of a sterile pyrogen free 0 9% sodium chloride solution Experiments on anaesthetized animals indicated that this infusion had only a slightly lowering effect on the blood pressure Oxygen consumption was recorded during the infusion and for a further 180 minutes Blood samples for lactic acid determination were taken at 20,
...es A basal lactic acid content of the blood

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Karlog O & E Knudsen Vitamin B_6 as an antidote against the rodent cide Castrix®(2 chloro 4 methyl 6 dimethylaminopyrimidine) *Nature* 1963 200 790
Lehman A J A report to the Association of Food and Drug Officials on current developments Part II Pesticides *Quart Bull Ass Food & Drug Officials* Oct 1951 122–133
Przygodda W Vogelwelt und Feldmausbekämpfung *Regulus Bull de la ligue luxembourgeoise pour l'etude et la protection des oiseaux* 1957 37 3–18



appreciably (fig 1) The effects reached a maximum 30 minutes after beginning the infusion, but still persisted at the end of 200 minutes In view of this dual stimulation, it was of interest to investigate the extent to which the augmentation of the oxygen consumption could be attributed to increased lactic acid oxidation An earlier study had demonstrated a relation between oxygen consumption as a percentage of the basal value (Y) and the lactic acid content of the blood in milligrams per cent (X), expressed by $Y = 11 2 \log X + 93 2$ (LUNDHOLM 1949) On the basis of this relation we calculated the degree to which increased oxidation of lactic acid might have been responsible for the elevated oxygen consumption (fig 2) The area below this theoretical curve was, however, only 45 per cent of that below the experimentally plotted curve It therefore seemed probable that chloroisoprenaline stimulated the oxygen consumption by some mechanism besides that of increased lactic acid oxidation

Both adrenaline and isoprenaline appreciably increased lactic acid production When administered after chloroisoprenaline, however, their effects appeared to be completely blocked, the blood lactic acid curve did not diverge from that associated with chloroisoprenaline alone Table 1 shows the blood lactic acid values on terminating the infusion of adrenaline and isoprenaline and also the differences between those

higher than 10 mg% was not tolerated. In other series of experiments we first infused chloroisoprenaline as described above and then, over a 60-minute period beginning 10 minutes after completion of the infusion, L-adrenaline 0.5 µg/kg/min. or DL-isoprenaline 0.5 µg/kg/min. Adrenaline and DL-isoprenaline (base) were dissolved in 20 ml sterile pyrogen-free 0.9% sodium chloride solution to which had been added 0.125 per cent ascorbic acid to prevent oxidation of the catecholamines. In these experiments also the oxygen consumption was determined for 180 minutes after beginning the adrenaline or isoprenaline infusion. Further series of experiments were conducted with infusions of 0.5 µg adrenaline or isoprenaline only. The lactic acid content of the blood was determined by the method of FRIEDEMANN & GRAESER (1933).

Results

Chloroisoprenaline by itself at a dose of 15 mg/kg stimulated both lactic acid production and oxygen consumption, though its effects varied

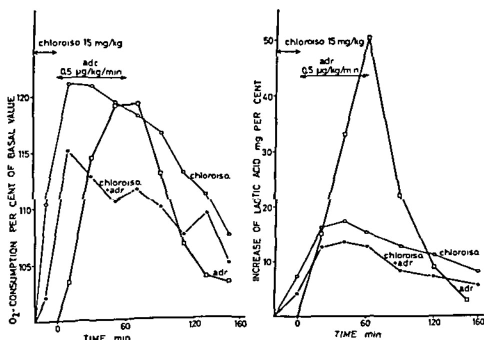


Table 2

Effects of chloroisoprenaline 15 mg/kg [illegible] adrenaline, 0 5 μg/kg/min , and of isoprenaline, [illegible]

	1 Chloroiso prenaline	2 Adrenaline	3 Isoprenaline	4 Chloroiso-prenaline + *Adrenaline*	5 Chloroiso-prenaline + *Isoprenaline*
	107 3	106 5	115 5	113 0	102 8
	111 6	114 6	104 4	104 4	116 0
	107 2	109 8	104 3	109 5	106 3
	101 0	117 8	107 5	105 6	104 3
	114 7	116 0	103 4	111 3	122 9
	111 7	111 2	102 6	106 3	106 7
	113 5	102 7		117 9	110 2
	121 9	108 6			117 5
	101 1	105 2			126 2
	125 3				127 4
	119 8				118 6
	140 2				122 1
	112 5				117 9
Mean ± S E M	*114 9* ± 3 07 P < 0 001	*110 3* ± 1 71 P < 0 001	*106 3* 1 96 P < 0 01	*109 7* ± 1 81 P < 0 001	115 3 ± 2.33 P < 0 001

Difference 1 + 2 − 4 = 15 5 ± 4 0 P < 0 001
1 + 3 − 5 = 5 9 ± 4 3 P < 0 2

the oxygen consumption curves for chloroisoprenaline with and without *isoprenaline strongly suggests that the effect of isoprenaline was blocked*

Discussion

It was clear from these experiments that chloroisoprenaline, though itself stimulating lactic acid production, blocked the effects of adrenaline and isoprenaline This behavior may suggests that competitive inhibition occurred and that chloroisoprenaline *stimulated the* pertinent receptors while blocking them for adrenaline and isoprenaline Such an inhibition of adrenaline's stimulatory effect on lactic acid production would be similar to that previously demonstrated for yohimbine (MOHME-LUNDHOLM 1956)

Even the calorigenic effects of adrenaline and isoprenaline were apparently blocked by chlorisoprenaline, though the latter drug stimulated the oxygen consumption so markedly that the inhibition was less evident In view of its own powerful effects on lactic acid production and oxygen consumption, chloroisoprenaline cannot be regarded as an ideal inhibitor of those effects

Table 1

[illegible]

60 min after that of adrenaline and isoprenaline

	1 Chloroiso prenaline	2 Adrenaline	3 Isoprenaline	4 Chloroiso prenaline + Adrenaline	5 Chloroiso prenaline + Isoprenaline
	12 9	74 6	41 0	28 1	23 3
	10 7	54 4	28 5	21 5	18 1
	24 9	39 4	44 2	17 7	12 2
	12 9	71 8	44 2	19 5	27 0
	10 7	58 9	33 8	10 0	19 2
	33 3	79 4	63 5	23 3	15 2
	42 2	44 7		19 5	13 4
	10 4	44 0			57 3
	5 9	43 1			15 0
	16 1				37 6
	32 2				26 9
	40 8				11 8
	31 0				13 2
Mean ± S E M	21 8 ± 3 5 P < 0 001	56 7 ± 5 1 P < 0 001	42 5 ± 4 9 P < 0 001	19 9 ± 2 1 P < 0 001	22 3 ± 3 6 P < 0 001

Difference 2–4 = 36 8 ± 5 5 P < 0 001
3–5 = 20 2 ± 6 1 P < 0 01

values and the corresponding figures from experiments in which the relevant infusion was preceeded by treatment with chloroisoprenaline The differences were statistically significant, thus demonstrating that chloroisoprenaline blocked the stimulatory action of both adrenaline and isoprenaline on lactic acid production

Since neither adrenaline nor isoprenaline reinforced the action of chloroisoprenaline alone on oxygen consumption, it seems likely that chloroisoprenaline also blocked their calorigenic effects On the other hand, the effect of chloroisoprenaline on oxygen consumption was so much greater than the effects of adrenaline and isoprenaline that the blockade was less evident than with lactic acid production With a view to demonstrating this blockade statistically, we analysed the results for any disparity between the sum of the separate effects of chloro isoprenaline and adrenaline and the effect of those two drugs in combination We found a difference of 15 5 ± 4 0 (P < 0 001), which was statistically significant In the experiments with isoprenaline the corresponding difference was 5 9 ± 4 3 (P < 0 2) Although the latter discrepancy was not statistically significant, the almost identical course of

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From the Biological Department, Ferrosan Ltd , Copenhagen.

Investigations into the Diuretic Effect and Elimination of Triamterene

By

J. Buus Lassen and Ole E. Nielsen

(Received November 6, 1963)

Triamterene (2, 4, 7 triamino 6 phenyl pteridine) is a new diuretic that increases the renal excretion of sodium, which has no effect on or reduces potassium excretion (WIEBELHAUS *et al* 1961) Owing to its mode of action the drug was at first taken to be an aldosterone antagonist (CROSLEY *et al* 1961, LARAGH *et al* 1961) Subsequent investigations showed, however, that triamterene preserved its diuretic action and characteristic effect on electrolyte excretion in patients with Addison's disease who were not under treatment with a mineralocorticoid (LIDDLE 1961), in adrenalectomized rats and in normal humans whose aldosterone production had been reduced by loading with sodium chloride (BABA *et al* 1962) HERKEN & SENFT (1961) could not show any natriuretic effect of triamterene in adrenalectomized rats, though they confirmed the effect on potassium retention

Our investigations were conducted to compare the diuretic action of the drug with that of chlorothiazide Further, we have studied the effect of triamterene on renal electrolyte and acid base excretions, as well as the elimination of the drug Finally, the renal clearance of triamterene has been examined The drug was tested as triamterene hydrochloride, which contains 80 per cent of the base

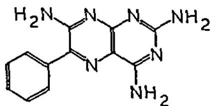


Fig 1 Triamterene

Summary

In experiments on rabbits, chloroisoprenaline at a dose of 15 mg/kg raised the oxygen consumption by up to 20 per cent and the lactic acid content of the blood by approximately 20 mg per cent. The effects persisted for at least three hours. Chloroisoprenaline blocked, simultaneously, the calorigenic and the lactic acid-stimulating effects of L-adrenaline at 0.5 µg/kg/min. and DL-isoprenaline at 0.5 µg/kg/min. administered by intravenous infusion over a 60-minute period.

Acknowledgement

The authors gratefully acknowledge the technical assistance of Mr Theodor Dzedin. Financial support has been given by the Medical Research Council. Chloroisoprenaline was kindly supplied by Dr. I. Slater of the Lilly Research Laboratories, Indianapolis.

REFERENCES

Friedemann, T. E & J B Graeser The determination of lactic acid *J biol Chem* 1933, 100, 291–308

Kennedy, B L & S Ellis Interactions of sympathomimetic amines and adrenergic blocking agents at receptor sites mediating glycogenolysis *Fed Proceed* 1963, 22, 449

Lundholm, L · The effect of adrenaline on the oxygen consumption of [illegible]

A Ciba Foundation symposium on adrenergic mechanism 1960, 303–321 London Churchill Ltd

Lundholm, L & N Svedmyr The effect of isoprenaline and adrenaline on oxygen consumption and lactic acid content of the blood in the rabbit *Acta physiol Scand* 1964, (in press)

[illegible]

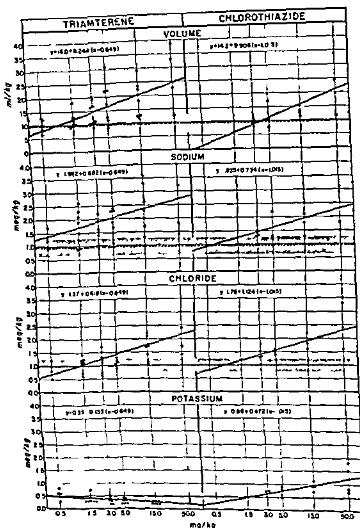


Fig 2 *Urine volume and electrolyte excretion in rats after oral administration of triamterene*

The excretion of this ion was found to fall with increasing doses of triamterene (dose response curve with a significant negative slope) and to rise with increasing doses of chlorothiazide (dose response curve with a significant positive slope)

Material and Methods

Diuretic experiments in rats The potencies of triamterene and chlorothiazide were compared in experiments on rats The two drugs were administered by stomach tube, suspended in 3 ml of saline 0 9% The control group was given 3 ml of saline alone The rats were placed in wire cages on funnels, four rats in each cage The urine was collected for 6 hr periods The animals were allowed to drink water freely but fasted for 18 hrs before as well as during the experiment Twelve experiments (144 rats) were conducted with triamterene and five (60 rats) with chlorothiazide The control group comprised 68 animals

Diuretic experiments on human subjects The diuretic effect and mode of action were studied in eight normal subjects The urine was collected for a 24 hr control period Triamterene was given orally, suspended in 200 ml of water Four doses were used 125 mg 250 mg, 375 mg and 500 mg During the 24 hr experimental period the urine was collected continously for two periods of 1 hr each, two of 2 hrs each, one of 4 hrs and one of 14 hrs Finally, the urine was collected for a 24 hr postexperimental period The glomerular filtration rate was measured by the endogenous creatinine clearance No special dietary measures were taken but the fluid intake of the subjects was as far as possible kept constant

Renal clearance experiments in pigs The renal clearance of triamterene has been compared with those of inulin and PAH in pigs Pigs (weighing 10–15 kg) had both ureters catheterised under pentobarbital anaesthesia The urine was then collected from each kidney A powerful diuresis was induced by infusing 3% sodium chloride The clearance technique, the usual one, involved administration of loading doses and then infusion of maintenance doses of the drugs tested Owing to the diuresis the individual clearance periods were short (3 min) Blood samples were withdrawn for every three clearance periods, and the serum values obtained showed stable conditions in the individual experiments

Analytical methods Sodium and potassium were determined by flame photometry, chloride by complexometric titration, bicarbonate manometrically (van Slyke) titrable acid by titration to pH 7 4 in the titrator, ammonia by Conway s microdiffusion method Creatinine, inulin and PAH were determined colorimetrically and triamterene fluorimetrically

Results

A *Diuretic Experiments*

1 Rats

The values illustrated in fig 2 show that triamterene has a diuretic effect almost four times greater than that of chlorothiazide, estimated on a weight basis (3 8 times, 95% confidence limits 1 4–10 0) Approximately the same potency was seen with the excretion of the sodium ion the basis for comparison (4 1, limits 1 0–16 6) The chloride excretion, on the other hand, was lower after triamterene than after chlorothiazide (0 7; limits 0 3–0 9) The dose-response curves for potassium show the characteristic difference between the modes of action of the two diuretics

within the 24-hr control period and the 24 hr experimental period Determinations of bicarbonate and titratable acid in the urine were only included in the four experiments with the largest triamterene doses

Fig 3 illustrates the hourly excretion of the ions mentioned and titratable acid, as well as the pH of the urine and the creatinine clearance, in a representative experiment in which the triamterene dose was 500 mg The diuretic effect is seen to have set in rapidly, to have reached a maximum within the first few hours and to have persisted for about 10 hours The excretion of sodium chloride and bicarbonate increased, but the excretion of potassium, ammonium and titrable acid decreased The urinary pH rose considerably The creatinine clearance showed slightly raised values Falling glomerular filtration rate was not seen

B *Elimination*

1 Human Experiments

In the human experiments with single doses of triamterene the urinary excretion of the drug was determined

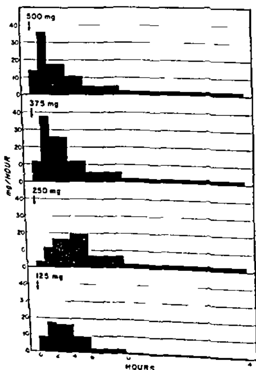


Fig 4 Excretion of triamterene in the urine after oral intake of different doses by four normal men

Table 1

Urinary volume and electrolyte excretion for eight normal men after oral administration of triamterene

Triamterene mg	Subject	URINE VOLUME ml/24 *hours*		SODIUM meq /24 hours		POTASSIUM meq /24 hours		CHLORIDE meq /24 hours		BICARBONATE meq /24 hours		AMMONIA meq /24 hours		TITRABLE ACIDITY meq / 24 hours	
		control period	exp period	control period	exp period	control period	exp period	control period	exp period	control period	exp period	control period	exp period	control period	exp period
125	I	1340	1940	146	221	51	47	134	164			40	31		
125	II	1060	1340	134	220	75	63	134	173			45	37		
250	III	1020	1490	122	204	53	25	133	151			47	30		
250	IV	1020	1750	146	292	76	63	162	231			45	36		
375	V	610	1225	52	161	61	42	55	124	2	9	25	19	26	17
375	VI	845	1450	171	232	56	44	162	184	5	13	33	22	15	6
500	VII	700	1030	43	111	35	29	38	66	3	14	35	27	18	8
500	VIII	1600	2145	264	318	83	44	234	267	10	30	53	48	16	5

2 Human Subjects

In table 1 are recorded the values found for the eight subjects' urine volume and excreted sodium, potassium, chloride and ammonium ions

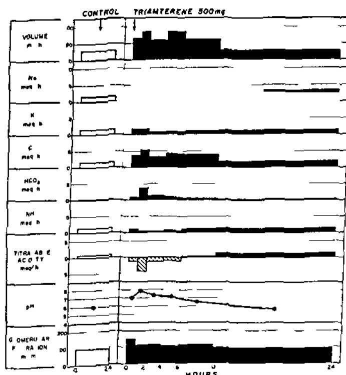


Fig 3 *Effect of triamterene (500 mg) on the urine volume and the composition of the urine in one normal man*

only from the significantly different slopes of the dose response curves for potassium, but also from their different effects on chloride excretion Both drugs increase bicarbonate excretion In chlorothiazide this property is attributable to its carbonic anhydrase inhibition, but the same is not true for triamterene, *in vitro* experiments showing it to have no effect on the carbonic anhydrase (BUUS LASSEN & NIELSEN 1963, unpublished observations) The influence of triamterene on renal acid base excretion has been further elucidated by experiments in man These results confirmed that the incomplete excretion of chloride relative to that of sodium ion is counterbalanced by increased bicarbonate excretion and diminished excretions of hydrogen ion (ammonia and titratable acid) and potassium ion, causing a pronounced rise in urinary pH

In these short term experiments on rats and human subjects no systematic alterations were demonstrated in the potassium content of the organism or in acid base balance

Summary

Triamterene (2, 4, 7 triamino 6 phenyl pteridine) is a diuretic increasing the excretion of sodium and depressing that of potassium On plotting dose response curves for comparative experiments with chlorothiazide in rats, the diuretic and natriuretic effects of triamterene were found to be about four times those of chlorothiazide The influence of triamterene on acid base excretion has been studied in short term experiments on human subjects The drug is eliminated by tubular excretion rather than by glomerular filtration, and about 30% of the amounts given orally are recovered in the urine within 24 hrs

Acknowledgements

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REFERENCES

Baba W J G R Tudhope & G H Wilson Triamterene a new diuretic drug *Brit med J* 1962, II 756–760

Catel [illegible]

Crosl [illegible]

Done [illegible]

Herken H & G Senft 2 4 7 triamino-6-phenylpteridin als "Aldosteron antagonist *Klin Wochenschr* 1961 39 1205–1206

Laragh J H E. B Reilly T B Stites & M Angers Pteridine compound as an inhibitor of aldosterone action in man *Fed Proc* 1961 20 410

Table 2

The calculated clearance values for each kidney in two experiments

	Left kidney clearance ml/min			Right Kidney clearance ml/min		
	Inulin	PAH	Triamter	Inulin	PAH	Triamter
Pig weight 11 kg	16	55	51	20	63	51
	20	61	58	19	71	56
	19	59	54	20	62	64
Pig weight 15 kg	19	45	39	20	53	49
	19	45	49	23	51	50
	19	47	54	25	51	50

Fig 4 illustrates the hourly renal excretions of triamterene in four experiments after different doses Maximum excretion occurred 2–4 hrs after administering the drug Within the first 24 hrs the excretion in the urine averaged 29% (16–39) of the dose given in the eight experiments and 3% (1–5) during the next 24 hrs These figures are in fair agreement with those of other workers (BABA *et al* 1962)

Ultrafiltration of plasma by the technique of LAUSEN (1955) has shown that *67% (64–70) of triamterene in human plasma is protein bound*

2 Renal Clearance Experiments in Pigs

The renal clearance of triamterene has been compared with those of inulin and PAH in pigs (table 2) The triamterene clearance greatly exceeded the inulin clearance, the values coming close to those for PAH clearance

The mean clearance values for all 26 periods were, for inulin 4 1 ± 1 8 *ml/min /kg, for PAH 12 6 ± 2 8 and for triamterene* 8 4 ± 3 4 (all values given with twice the standard deviation) The results of these trials showed triamterene to be eliminated mainly by tubular excretion

Discussion

The characteristic effect on renal electrolyte excretion that triamterene possesses by increasing sodium excretion and depressing that of potassium was most plainly *visible during the comparative investigations with* chlorothiazide in rats Further, the dose response curves of the two diuretics show *that triamterene* is four times more active than chlorothiazide, assessed by its natriuretic and diuretic effects, on a weight basis The different mechanisms of action of the two drugs are evident not

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From the Departments of Histology and Pharmacology, University of Lund, Lund, Sweden

The Direct Demonstration of a Barrier Mechanism in the Brain Capillaries

By

Åke Bertler, Bengt Falck and Evald Rosengren

(Received January 25, 1964)

It is well known that the catecholamines and 5 HT[1]) are effectively prevented from passing the blood brain barrier but the structure that constitutes the blocking membrane is not known. By combining the methods now available for the detection of these amines and their precursors at the cellular level (FALCK 1962, FALCK *et al* 1962) it is possible to attack this problem directly.

Methods

Mice and rats were used in this investigation. The distribution of fluorescent material was studied in various parts of the brain (mainly the cerebellum) and in a peripheral preparation containing the larynx and the upper trachea together with the surrounding [illegible]

were administered. In other experiments a minute amount of a solution of L-DOPA or DA was deposited in the cerebellar parenchyma by means of the finest injection needle. The animals were beheaded either 20 or 60 min after the last injection. The amines and their precursors in the different structures taken for examination were made visible by the specific and sensitive fluorescence microscopic method of FALCK (1962, 1964). These compounds were also determined chemically by the methods of BERTLER *et al* (1958) and BERTLER & ROSENGREN (1959a).

Results and Discussion

After administration of L-DOPA (100 mg/kg) intraperitoneally, a weak green fluorescence developed in the capillary endothelium of the brain

[1]) Abbreviations used: 5 HT = 5 hydroxytryptamine, NA = noradrenaline, DA = dopamine, DOPA = d hydroxyphenylalanine, 5 HTP = 5 hydroxytryptophan, MAO = monoamine oxidase.

Lassen J B & O E Nielsen *Unpublished investigations*, 1963
Lausen, H A simple method for ultrafiltration through cellophane *Acta pharmacol et toxicol* 1955, 11, 353–354
Liddle, G W Specific and non specific inhibition of mineralocorticoid activity *Metabolism* 1961, 10, 1021–1030
Wiebelhaus, V D, J Weinstock, F T Brennan G Sosnowski & T J Larsen A potent, non steroidal orally active antagonist of aldosterone *Fed Proc* 1961, 20, 409
Wilson, G M Diuretics *Brit med J* 1963, I, 285–292

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Methods

Mice and rats were used in this investigation. The distribution of fluorescent material was studied in various parts of the brain (mainly the cerebellum) and in a peripheral preparation containing the larynx and the upper trachea, together with the surrounding musculature and the thyroid gland after the injection of L-DOPA, D-DOPA, DA, 5 HTP or 5 HT [illegible] were administered. In other experiments [illegible] or DA [illegible] needle [illegible] amines and their precursors in the different structures taken for examination were made visible by the specific and sensitive fluorescence microscopic method of FALCK (1962, 1964). These compounds were also determined chemically by the methods of BERTLER *et al* (1958) and BERTLER & ROSENGREN (1959a).

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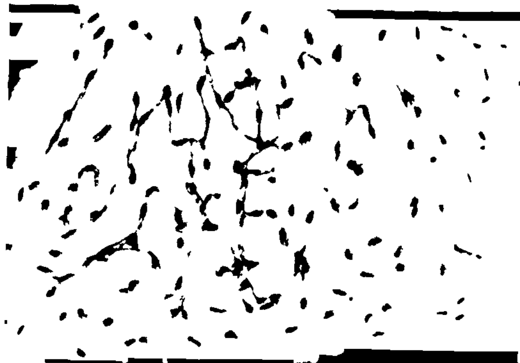


Fig 1 The intensely fluorescent capillary endothelium in the mouse brain after MAO inhibition and administration of L DOPA The animal was first injected with nialamide (100 mg/kg) and one hr later with L DOPA (100 mg/kg) and was killed 20 min later Magnification 250 ×

preparations treated for histochemical examination Except for the adrenergic neurons, which displayed high fluorescence, the brain parenchyma appeared dark Nor could any fluorescence be observed in the walls of the larger vessels within the brain, in contrast to the meningeal vessels, which emitted a green light In the peripheral tissues the injection of L-DOPA did not cause any accumulation of fluorescent material in the capillaries Here the fluorescent material passed out of the blood vessels and accumulated in various structures This striking difference between the peripheral and the intracerebral capillaries was best demonstrated in the eye only in the retina did the capillary endothelium fluoresce after administration of L-DOPA

The endothelial fluorescence in the brain capillaries induced by treatment with L-DOPA was dramatically increased after inhibition of MAO (Fig 1) This seems to imply that the fluorescent product was at least in part derived from DA This view finds further support from the fact that, after nialamide treatment and injection of L-DOPA, huge amounts of DA but no DOPA were found spectrophotofluorimetrically in the cerebellum No significant amounts of DOPA or DA could be detected in untreated animals

In the cerebellum of the animals that received an injection of L-DOPA directly into the cerebellum, a spot with enormous diffuse fluorescence was found at the endpoint of the stich channel The fluorescent area was not sharply delineated, the fluorescence showed a gradual decrease outwards towards the non fluorescent parenchyma Numerous capillaries displayed high endothelial fluorescence in the fluorescent area as well as in adjacent areas of non fluorescent parenchyma

In contrast to the findings after the administration of L DOPA, no fluorescence could be observed in the brain capillary endothelium even after injection of huge doses of DA (500 mg/kg) Nor did pre treatment of the animals with a MAO inhibitor before the administration of DA produce capillary fluorescence However, in areas known to be located outside the blood brain barrier (cf JEPPSON 1962), e g the median eminence, many capillaries were seen to be loaded with fluorescent material In these regions an unmistakable parenchymal fluorescence also appeared Thus, in one and the same section, brain capillaries that were permeable and those that were impermeable to DA could be demonstrated In the periphery, systemic administration of DA produced strong, diffuse fluorescence in the tissues, the intensity was of a magnitude that made it impossible to distinguish the walls of smaller vessels from other structures After intracerebral injection of DA, the depot and an area round it showed intense fluorescence, which gradually decreased towards surrounding dark parenchyma In this region, the capillary network appeared dark, and no fluorescent endothelial cells could be observed Thus, DA does not seem to enter the endothelium of the brain capillaries from either side

The findings, that DA does not penetrate the capillary walls, but that after administration of L-DOPA a fluorescent substance – which for reasons mentioned above seemed to be derived mainly from DA – occurred in the capillary endothelium, imply that the amine had been formed within the endothelium Consequently, this must contain DOPA decarboxylase The correctness of this deduction was confirmed by the finding that after inhibition of DOPA decarboxylase, treatment with L-DOPA gave rise to a diffuse fluorescence throughout the whole mass of the brain, whereas the capillary walls showed only a faint fluorescence and that, concomitantly, considerable amounts of DOPA, but no DA, were detected spectrophotofluorimetrically Further, after combined inhibition of DOPA-decarboxylase and MAO, L-DOPA administration resulted only in poor capillary fluorescence From the observat[illegible]
L-DOPA [illegible]
to D[illegible]
amir[illegible] [illegible]ss of this barrier cannot at present be evaluated It is clearly established, however, that L-DOPA administered at relatively

high dose levels has effects on central neurons (CARLSSON *et al* 1957) After injection of large doses of L-DOPA, BERTLER & ROSENGREN (1959a) demonstrated a high synthesis of DA in brain areas known to be rich in adrenergic structures, *e g* the caudate nuclei and the hypothalamus, but only slight formation of DA in the cerebellum, whereas in our study the distribution of fluorescent capillaries seemed to be uniform in different brain parts The only reasonable conclusion to draw from these facts is that, when injected in sufficient amounts, L-DOPA passes to some extent the barrier and is decarboxylated in the parenchyma

The experiments in which 5-HTP and 5-HT were used with or without previous inhibition of MAO indicated that these compounds behaved in the same way as did L-DOPA and DA, respectively

D-DOPA administration gave essentially the same results as those obtained after DA injection, except that few endothelial cells exhibited a weak fluorescence The possibility cannot be excluded that this reflects an uptake of D DOPA It should, however, be kept in mind that the D DOPA might have been contaminated with L DOPA or that L-DOPA can be formed in the body from the D analogue

An unexpected finding was that the cerebellum of the mouse contains relatively high amounts of NA – 0 5 μg/g – compared with that of other species (VOGT 1957, BERTLER & ROSENGREN 1959b) Histochemically the only structure having the fluorescence characteristics of primary catecholamines (cf FALCK 1962) was found to be varicose nerves These occurred in an amount that seemed to correspond well to the amount of NA

Summary

It has been demonstrated that the vascular endothelium in the central nervous system constitutes a barrier for D DOPA and dopamine between the blood and brain parenchyma Further the brain capillaries were found to contain DOPA decarboxylase and MAO and thus also forms an obstacle for the penetration of the L-isomers of DOPA and 5-HTP

Acknowledgements

This work has been supported by grants from Marta and Ingemar Ekbloms Foundation, United States Public Health Service (NB 02854-04) and the Swedish Medical Research Council

REFERENCES

Bertler Å, A Carlsson, E Rosengren & B Waldeck A method for the [illegible]

382–384

[illegible]

Falck, B Observations on the possibilities of the cellular localization of monoamines by a fluorescence method *Acta physiol scand* 1962, 56 Suppl 197

Falck B Cellular localization of monoamines *Progress in Brain Research* 1964 (in press)

Falck, B, N-Å Hillarp, G Thieme & A Torp Fluorescence of catechol amines and related compounds condensed with formaldehyde *J Histochem Cytochem* 1962, 10, 348–354

Jeppson, P G Studies on the blood brain barrier in hypothermia *Acta neurol scand* 1962, 38 Suppl 160

Vogt, M Sympathomimetic amines in the central nervous system *Brit Med Bull* 1957, 13, 166–171

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Acknowledgements

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III The Secondary Urinary Kinin (Z_2) Concentrates of Z_2 were prepared as previously described (BRISEID JENSEN & VENNEROD 1962 c) A highly purified prep-

added double strength Jalon s solution and then normal Jalon s solution for the rat uterus assay The other portion was diluted in the same way with Tyrode solution for the guinea pig ileum assay The assays were carried out immediately and simultaneously on both biological preparations

B *Pharmacological Assays*

For the pharmacological parallel assays the biological effects used were stimulation of the isolated rat uterus stimulation of the isolated guinea pig ileum rat blood depressor effect and increased capillary permeability in guinea pigs The techniques employed for the two first mentioned biological effects were those previously described (BRISEID JENSEN & VENNEROD 1962 b) (2 + 2) or ' bracketing assays being used The fractions from the paper chromatograms were estimated in rat uterus ' bracketing ' experiments, the standard dose ratio usually being 3 2

The rat blood pressure experiments were partly carried out as described in the above mentioned paper as direct depressor assays, partly also as adrenaline inhibition tests, a suitable adrenaline dose was injected repeatedly into the jugular vein until the blood pressure responses were stable Then bradykinin standard and Z_2

sometimes 4 adrenaline doses were given to obtain stable blood pressure responses In some experiments a ganglion blocking agent, chlorisondamine chloride=Ecolid ® 0 1 mg/100 g was used

For *the capillary permeability test* the method of MILES & MILES (1952) was used with minor modifications A 2% w/v solution in distilled water of Evans blue was injected intravenously into the legs of guinea pigs weighing 300–400 g, 0 05 ml/100 g Solutions of the kinins in 0 9% sodium chloride in distilled water were injected 20–30 minutes later intradermally into the abdominal skin After 10–15 minutes the mean diameter of blueing was measured. To each animal were administered in a randomized order four doses of standard and four of test solution, the dose ratios within the series being 1000 100 10 1 The straight log-dose response curves were drawn and the activity of the unknown preparation roughly estimated Sometimes the highest concentration injected gave a result falling obviously outside the curve, or the lowest concentration injected gave no blueing the curve was then based on 3 doses only Two three or four animals were used to obtain each result recorded here

C *Chromatography*

Paper chromatography with n butanol acetic acid and water (40 + 10 + 50 vol) was carried out as previously described (BRISEID JENSEN & VENNEROD 1962 b) Quantities of Z_2 corresponding to 2-5 µg of bradykinin when tested on the rat uterus were applied on the chromatograms The 2 µg runs were used for localizing the active zones the 5 µg runs for isolation purposes

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From the Biological Department, the Pharmaceutical Institute
University of Oslo

Pharmacological Properties of Human Urinary Kinins

By

Kjell Briseid Jensen, Anne Marie Vennerød and Olav Kjell Dyrud

(Received November 14 1963)

In previous work a comparison has been made between bradykinin and a fraction of the kinins isolated from normal human urine (BRISEID JENSEN & VENNERØD 1962 c) The fraction examined was called Z_2, to distinguish it from a fraction (Z_1) which could be differentiated from bradykinin neither in chromatographic nor in pharmacological parallel tests (BRISEID JENSEN & VENNERØD 1962 b) Z_2 was found to be significantly more potent than bradykinin both in stimulating the isolated guinea pig ileum and in rat blood pressure assays, compared with the effect on rat uterus The blood-pressure observations were not surprising, as preparations containing both Z_1 and Z_2 had previously been found slightly more active than bradykinin in rat blood pressure assays No such observations, however, had been made in guinea pig ileum experiments

In this paper we describe tests on new batches of Z_2 for their effects on rat blood pressure, their stimulation of guinea pig ileum and their ability to increase capillary permeability in guinea pigs Comparisons were made with bradykinin, the isolated rat uterus being used throughout as biological reference preparation The blood pressure experiments were carried out partly as direct *depressor assays, partly as indirect* assays based on adrenaline inhibition In some of the experiments a ganglion blocking agent was injected For the guinea pig ileum experiments the quantities required of Z_2 were so small that they could be obtained from the concentrates by further chromatographic purification on paper and elution of the active spots

Technique

A *Preparations of the Kinins*

I Bradykinin Synthetic bradykinin (Sandoz) which was available in ampoules containing 100 µg/ml was used

II The Main Urinary Kinin (Z_1) Concentrates of Z_1 were prepared as previously described (BRISEID JENSEN & VENNERØD 1962 a)

Table 2

Z_2-preparation	ng bradykinin (Index of discrimination)	Fiducial limits %, P = 0 05	
		Uterus	Ileum
1*)	15	81–124	93–107
3	1 4	–	–
3 (purified by paper chromatography)	0 3	–	–
4	2 3	–	–
5	2 2	–	–
5 (purified by paper chromatography)	0 7	–	–

*) An index of 11 9 was previously found for the same Z_2 preparation (BRISEID JENSEN & VENNERØD 1962 c)

tained in (2 + 2) assays with 6 series of 4 randomized doses The statistical procedure described by SCHILD (1942) was used for treatment of the (2 + 2) assays The calculation procedure for the capillary permeability assays was the one described above

From the tables it can be seen that 5 different Z_2 preparations have been examined As the capillary permeability test has not been used in previous work on the main urinary kinin (BRISEID JENSEN & VENNERØD 1962b), a Z_1 preparation previously tested for other biological properties was also compared with bradykinin for its capillary effect

Table 2 shows that *one Z_2-preparation* tested previously for its guinea pig ileum effect (BRISEID JENSEN & VENNERØD 1962 c) gave about the same significant index of discrimination However, three new batches prepared by the same method could not be differentiated from bradykinin by *simultaneous assays on rat uterus and guinea pig ileum* The possibility exists that ileum inhibiting impurities in the new Z_2 preparations are responsible for these results However, the index for two of the preparations further purified by paper chromatography was somewhat lower, and it seems more probable that a low index is correct and that contaminants in Z_2-preparation 1 were responsible for the higher index observed

As previously pointed out (BRISEID JENSEN & VENNERØD 1962 *c*), *not only Z_2 preparation 1, but also* another batch of Z_2 prepared from other urines by a different procedure (hydrochloric acid elution of the Amberlite CG 50 instead of sodium hydroxide elution), gave a high discrimi-

Comments on Technique

A Capillary Permeability Tests

The capillary permeability test with unanaesthetized guinea pigs is only roughly quantitative In order to evaluate the precision of the method, as employed by us we arranged for the assay of a bradykinin solution of strength unknown to the observer Twelve animals were used, of which 2 had to be discarded because of un successful intradermal injections giving too diffuse blueing areas The bradykinin standard solutions contained 10, 1, 0 1, and 0 01 µg/ml, and the test solutions were adjusted to give effects similar to those of the standards The diameters of the blueing areas ranged from 3 5 to 9 0 mm in both the standard and the test series The log dose response curves were drawn by eye, the results were calculated from the distance between the curves and are shown in table 1 From the results it can be calculated that the value obtained from 4 guinea pigs should give a standard error of about 25% and consequently confidence limits of ± about 60% (P = 0 05)

Table 1.

Capillary permeability assay, precision of method
Assay of a bradykinin solution containing 1000 ng/ml
For details see text

Animal	Observed bradykinin concentration ng/ml
1	1780
2	520
3	2050
4	800
5	2230
6	930
7	520
8	1080
9	1660
10	1930
Mean	1350
Standard deviation	650
Standard error of the mean	206

Results

Results of comparative assays of the secondary urine kinin, Z_2, and bradykinin on different biological preparations are given in tables 2, 3 and 4 Unless otherwise stated the results from the guinea pig ileum experiments, the rat uterus experiments and the rat blood pressure experiments were based on repeated "bracketing" assays Some results of comparative guinea pig ileum and rat uterus determinations were ob-

adrenaline, the high index recorded for preparation 5 was considerably reduced A ganglion blocking agent was used in the adrenaline inhibition method to bring the blood pressure to a sufficiently low and stable level The ganglion blockade, however, was not responsible for the reduction in index, as experiments carried through without the blocking agent gave closely similar results In some experiments the index of discrimination was first determined by the direct depressor method without ganglion blockade, the index being then estimated by that method or by adrenaline inhibition after the ganglion-blocking agent had been injected

Discussion

The results reported above support the previous demonstration (BRISEID JENSEN & VENNERØD 1962 c) that the secondary urine kinin Z_2 can be distinguished from bradykinin both by paper chromatography and by pharmacological parallel assays However, it seems likely that of the effects examined only the combined rat blood depressor and rat uterus stimulation effects can be used safely for such a differentiation, and that the previous guinea pig ileum results (BRISEID JENSEN & VENNERØD 1962 c) could have been due to interfering substances present in some Z_2-concentrates

Besides bradykinin, the decapeptide kallidin is released in blood and might be thought to escape into the urine to some extent Although the possibility exists that both Z_1 and Z_2 are produced in the kidneys and that they may differ from bradykinin and kallidin, it seems justifiable from our results to conclude that Z_1 is identical with bradykinin and Z_2 with kallidin STÜRMER & BERDE (1963) have published results obtained with synthetic bradykinin and kallidin which show that the index of discrimination of guinea pig ileum/rat uterus is about 0 6, that of rat blood pressure/rat uterus about 5 5, and that of guinea pig capillary permeability/rat uterus about 1 7 From tables 2, 3 and 4 it can be seen that the indices mentioned agree well with the indices obtained with the purest Z_2 preparations

The paper chromatographic properties of Z_1 and Z_2 also seem to suggest that they may be identical with bradykinin and kallidin PLESS, STÜRMER, GUTTMAN & BOISSONNAS 1962) reported that they obtained R_f values of 0 28 and 0 15 for bradykinin and kall[illegible]d[illegible]n respect[illegible] the liquid system n butanol, acetic [illegible] found values of 0 34 and 0 19 for [illegible] We have noticed somewhat similar results for bradykinin and Z_2 (BRISEID JENSEN & VENNERØD 1962 c) in the same liquid system and have

Table 3

Pharmacological parallel assays
Guinea pig capillary permeability
Ng bradykinin equiactive with the amount of Z_2 corresponding to 1 ng of bradykinin when tested simultaneously on rat uterus

Z_2 preparation	ng bradykinin (Index of discrimination)	Number of guinea pigs
2	0 7	4
3	2 0	2
4	1 5	2
Z_1 preparation	1 3	4

nating index This suggests that the interfering impurities will sometimes be present in urine and that their behaviour during the purification process is so similar to that of Z_2 that the method of purification should be improved

Table 3 shows that neither Z_1, nor Z_2 can be distinguished from bradykinin by the capillary permeability test

The results in table 4 give support to the previous observation (BRISEID JENSEN & VENNERØD 1962 b, c) that Z_2 has a stronger rat blood depressor/rat uterus effect than do Z_1 and bradykinin The sizes of the indices found varied somewhat from one batch of Z_2 to another, suggesting that different amounts of contaminents could also affect the results The Z_2-preparation 5 especially gave a high rat blood depressor/rat uterus stimulation index When the direct depressor measurements were replaced by a method based on inhibition of the pressure effect of

Table 4

Pharmacological parallel assays
Rat blood depressor effect
Ng bradykinin equiactive with the amount of Z_2 corresponding to 1 ng of bradykinin when tested simultaneously on rat uterus
The figures in brackets indicate number of experiments when more than one assay was carried out
For details see text

Z_2 preparation	ng bradykinin (Index of discrimination)			
	Depressor method		Adrenaline inhibition method	
		ganglionic blockade		ganglionic blockade
3	10	–	–	–
4	7 (4)	6	6	4
5	17 (2)	15	6	5

adrenaline, the high index recorded for preparation 5 was considerably reduced A ganglion blocking agent was used in the adrenaline inhibition method to bring the blood pressure to a sufficiently low and stable level The ganglion blockade, however, was not responsible for the reduction in index, as experiments carried through without the blocking agent gave closely similar results In some experiments the index of discrimination was first determined by the direct depressor method without ganglion blockade, the index being then estimated by that method or by adrenaline inhibition after the ganglion blocking agent had been injected

Discussion

The results reported above support the previous demonstration (BRISEID JENSEN & VENNEROD 1962 c) that the secondary urine kinin Z_2 can be distinguished from bradykinin both by paper chromatography and by pharmacological parallel assays However, it seems likely that of the effects examined only the combined rat blood depressor and rat uterus stimulation effects can be used safely for such a differentiation, and that the previous guinea pig ileum results (BRISEID JENSEN & VENNEROD 1962 c) could have been due to interfering substances present in some Z_2-concentrates

Besides bradykinin, the decapeptide kallidin is released in blood and might be thought to escape into the urine to some extent Although the possibility exists that both Z_1 and Z_2 are produced in the kidneys and that they may differ from bradykinin and kallidin, it seems justifiable from our results to conclude that Z_1 is identical with bradykinin and Z_2 with kallidin STURMER & BERDE (1963) have published results obtained with synthetic bradykinin and kallidin which show that the index of discrimination of guinea pig ileum/rat uterus is about 0 6, that of rat blood pressure/rat uterus about 5 5, and that of guinea pig capillary permeability/rat uterus about 1 7 From tables 2, 3 and 4 it can be seen that the indices mentioned agree well with the indices obtained with the purest Z_2 preparations

The paper chromatographic properties of Z_1 and Z_2 also seem to suggest that they may be identical with bradykinin and kallidin PLESS, STÜRMER, GUTTMAN & BOISSONNAS 1962) reported that they obtained R_F-values of 0 28 and 0 15 for bradykinin and kall
the liquid system n butanol, ac
found values of 0 34 and 0 19
We have noticed somewhat similar results for bradykinin and Z_2 (BRISEID JENSEN & VENNEROD 1962 c) in the same liquid system and have

previously found Z_1 to run together with bradykinin (BRISEID JENSEN & VENNERØD 1962 b)

Although the above assumption of identity between Z_1 and bradykinin is based on direct pharmacological and chromatographic comparisons between Z_1-concentrates and synthetic bradykinin, the assumption of identity between Z_2 and kallidin is indirect and based on data from literature Consequently, a comparison of pure kallidin and a highly purified Z_2-preparation should be carried out before a final conclusion is reached

Summary

A comparison has been made between synthetic bradykinin (Sandoz) and concentrates of a kinin fraction (Z_2) from human urine The two preparations could be distinguished from each other in rat blood pressure/rat uterus parallel assays

Parallel tests, with the rat uterus on one hand and guinea pig ileum or guinea pig capillary effect on the other, both failed to differentiate between the two substances

The results show that the secondary human urine kinin fraction (Z_2) is not identical with bradykinin The indices of discrimination obtained in pharmacological parallel assays agree well with the values given in the literature for the decapeptide kallidin compared with bradykinin

Acknowledgements

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REFERENCES

Jensen K Briseid & A M Vennerød Purification of the main kinin of [illegible] 19 265 274

Jens [illegible] ological and chromato [illegible] urine with bradykinin

Jens [illegible] logical and chromatographic differentiation between a secondary kinin from human urine and bradykinin *Acta pharmacol et toxicol* 1962 c 19 337 344

Miles A A & E M Miles Vascular reactions to histamine histamine liberator and leukotaxine in the skin of guinea pigs *J Physiol* 1952 118 228 257

Pierce J V & M E Webster Human plasma kallidins Isolation and chemical studies *Biochem Biophys Res Comm* 1961 5 353 357

Pless J E Sturmer St Guttman & R A Boissonnas Kallidin Synthese [illegible] 1962 45 394–396

[illegible] biological assay on a preparation [illegible] illustrated by the estimation of histamine [illegible] 130

Sturmer E & B Berde A pharmacological comparison between synthetic bradykinin and synthetic kallidin *J Pharmacol* 1963 139 38–41

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From the Biological Department, the Pharmaceutical Institute, University of Oslo

Purification of a Secondary Kinin from Human Urine; A Pharmacological and Chromatographic Comparison with Kallidin

By

Kjell Briseid Jensen, Sidsel Flem Rinvik and Anne Marie Vennerød

(Received November 14 1963)

A method has previously been described for preparing a concentrate of a secondary kinin (Z_2) from human urine (BRISEID JENSEN & VENNERØD 1962 c) Different batches prepared by that method, however, were found to differ quantitatively to some extent in pharmacological parallel assays with bradykinin (BRISEID JENSEN, VENNERØD & DYRUD 1964) In the work reported here the method was therefore modified to give preparations of higher purity

The results obtained with some concentrates of Z_2 prepared by the original method, as well as with concentrates further purified by paper chromatography (BRISEID JENSEN, VENNERØD & DYRUD 1964), suggested that Z_2 might be identical with kallidin Pharmacological and other properties of that peptide seem now well established (PLESS, STÜRMER, GUTTMAN & BOISSONNAS 1962, STÜRMER & BERDE 1963) Concentrates of Z_2 prepared by the modified method described in this paper were accordingly compared with kallidin, both by paper chromatography and in pharmacological parallel tests

Technique

A *Preparations of the Kinins*

I Bradykinin Synthetic bradykinin (Sandoz), which was available in ampoules containing 100 µg/ml was used

II Kallidin Synthetic kallidin (Sandoz) which was available in ampoules containing 100 µg ml, was used

III The secondary urinary kinin (Z_2) Concentrates of Z_2 were prepared by the method previously described (BRISEID JENSEN & VENNERØD 1962 c) modified as described below The first stages of the purification procedure were those described previously for a 50-litre batch of urine When the amount of the [illegible]

(Z_1) present [illegible]

from wat [illegible]

(without adjusting the pH before the second extraction), considerable amounts of Z_2 were left in the water phase The further procedure was in principle parallel to that used for the preparation of Z_1 concentrates (BRISEID JENSEN & VENNERØD 1962 a)

The active solution (500 ml) was acidified to pH 1 5 by adding hydrochloric acid and then shaken each of three times with 500 ml of butanol In this procedure the pH was adjusted to 1 5 also before the second and the third extractions The residual water phase was discarded, and the combined butanol fractions were extracted with 3 × 500 ml of distilled water The combined water fractions were concentrated to dryness at 30° under reduced pressure (5–10 mm Hg) The active substance was eluted with 20 ml and 4 × 10 ml of concentrated acetic acid The combined acid fractions were filtered through filter paper, and the acid was removed on a rotating evaporator under a similar low pressure The concentrate was kept over silica gel in evaporated desiccators at room temperature

B *Pharmacological Assays*

For the comparative assays several pharmacological effects were used stimulation of the isolated rat uterus, stimulation of the isolated guinea pig ileum, rat blood depressor effect The techniques and the calculation of the results were those previously described (BRISEID JENSEN & VENNERØD 1962 b, BRISEID JENSEN, VENNERØD & DYRUD 1964) Only direct rat blood pressure determinations were employed and not the adrenaline inhibition method, which has been alternatively used by BRISEID JENSEN, VENNERØD & DYRUD (1964)

C *Chromatography*

Paper chromatography with n butanol, acetic acid and water was carried out as described previously (BRISEID JENSEN & VENNERØD 1962 b, BRISEID JENSEN, VENNERØD & DYRUD 1964)

Comments on Technique

A *Preparations of the Kinins*

In the modification of the method for preparing concentrates of Z_2, butanol extractions were incorporated in order to obtain a degree of purity of the final product comparable with that of the Z_1 concentrates (BRISEID JENSEN & VENNERØD 1962 a) It was pointed out in the previous paper that practically all Z_1 present in the acidified eluate from Amberlite CG 50 would pass over into the first butanol extract whereas Z_2 could only partly be transferred

It has also previously been established (BRISEID JENSEN & VENNERØD 1962 c) that nearly half the activity left in the sodium chloride saturated aqueous phase after butanol extraction at pH 1 5 (Z_2) could be shaken into butanol if hydrochloric acid was again added to give a pH of 1 5 In a model experiment 32% was transferred (47% of total regained activity), 36% was left in the water phase, and 32% was lost by inactivation It was assumed that the losses of activity were the same both for transferred and residual Z_2 However, later experience with Z_2 isolation suggested that a somewhat lower percentage of Z_2 than 47 was obtained under the conditions chosen The results of an isolation experiment given in table 1 thus agree with a transfer of about 32% of total regained activity for each butanol extraction together

Table 1.

Stages of purification	μg	Activity % recovery referring to	
		Eluate from Amberlite CG 50	preceding stage
1 [illegible]	1300	100	100
2 [illegible] (2x [illegible]	1250	96	96
[illegible]	(750)	(58)	(60)
3 [illegible]	235	18	19
4 [illegible]	125	10	53
(4x [illegible]	(55)	(4)	(23)
5 Combined water extracts from butanol extracts *b c d* concentrated to dryness acetic acid extracts of the residue concentrated to dryness Z_2	106	8	85

with an over all 24% loss of activity It may be of some significance for the comparison that the 47% transfer reported in the earlier paper (BRISEID JENSEN & VENNEROD 1962 c) was obtained by a repeated butanol extraction whereas the calculated 32% transfer here refers to a single butanol extraction, but there was no pH adjustment before the repeated butanol shaking To re-examine the Z_2-distribution, a

Table 2

Recoveries of Z_2 and bradykinin by butanol extraction from water

Test solutions

A 500 ml water stationary phase after butanol [illegible]

B [illegible]

Test solution	Activities				
	Start	Transferred to butanol		Left in water phase	
	μg	μg	%	μg	%
A	47	14 9	32	28 6	61
B	44	43	98	–	–

model experiment was carried out The results are given in table 2, which also shows the transfer of bradykinin under the same conditions

The test solutions (500 ml) were each shaken twice with 500 ml butanol The transferred activities were recovered from the combined butanol fractions by shaking three times with 350 ml of water each time Portions of 2 ml for assay were con centrated to dryness at 30° and 5–10 mm Hg on a rotating evaporator and then dried at 0 01–0 05 mm Hg at room temperature

Table 2 shows a transfer of about 34% for Z_2 of total regained activity, this is in good agreement with the results of table 1 The total loss of activity amounted to 7% As previously observed, (BRISEID JENSEN & VENNERØD 1962 a, c) all the brady kinin was transferred to the butanol

Table 1 shows an example of a purification experiment of Z_2 carried through with a 50-litre batch of urine Recoveries are given for different stages of the process calculated on the basis both of the original total activity of Z_1 and Z_2 in the eluate from the ion exchange and of the activity of the preceding stage From the table it can be calculated that, of the total amount of activity present in the eluate from the ion exchange, roughly ⅓ must have been due to Z_2, assuming that both substances are equally active against rat uterus

Results

A *Pharmacological Assays*

Results of comparative assays of Z_2 and kallidin on different biological preparations are given in table 3 Three different batches of Z_2 were examined The biological effects used in parallel were those that had previously differentiated between bradykinin and Z_2 (BRISEID JENSEN & VENNERØD 1962 c, BRISEID JENSEN, VENNERØD & DYRUD 1964)

Table 3

Pharmacological parallel assays

Ng kallidin equiactive with the amount of Z_2 corresponding to 1 ng kallidin when tested simultaneously on rat uterus

Biological effect	ng kallidin (index of discrimi nation)	Batch	Fiducial limits % P – 0 05	
			Rat uterus	–
Guinea pig ileum stimulation	0 4	1	96–104	95–105
	0 4	1	94–107	94–107
	0 2	2	91–109	93–107
	0 2	3	93–108	92–108
Rat blood depressor effect	1 7	1	*)	*)
	2 2	1	*)	*)
	1 1	2	91–109	*)
	0 8	3	93–108	*)

*) Result based on bracketing assays

B *Chromatography*

Tables 4 and 5 give results from paper chromatographic runs of Z_2, kallidin and a mixture of the two In the experiment referred to in table 4 a pure Z_2 preparation prepared by the method described in this paper was used, a Z_2 preparation containing some Z_1 was used for the experiment mentioned in table 5 The results in table 4 are given as ng recovered polypeptides and those in table 5 as percentage recoveries, in both tables calculated as kallidin and based on rat uterus assays Values for recoveries less than 1 per cent have been omitted

Table 4

Paper chromatography of kallidin and Z_2
Absence of Z_1 in Z_2
Liquid system N butanol acetic acid water (40 + 10 + 50 vol.)
Applied Z_2 in a single run Z_2 + kallidin (60 + 40%) in a mixed run
The quantities applied and the recovery values refer to kallidin standard in rat uterus assays
Temperature 23-24 C
Chromatogram run for 14 hours
Length of run 27 cm

Fraction number	Recovery ng	
	Z_2	Z_2 + kallidin
1	-	-
2	-	-
3	-	-
4	-	-
5	-	-
6	350	750
7	600	2200
8	900	120
9	-	-
10	-	-
11 20	-	-

Comments of Results

A *Pharmacological Assays*

1 Rat blood pressure The comparative rat blood pressure and rat uterus assays did not differentiate between Z_2 and synthetic kallidin For the 3 batches of Z_2 examined the indices of discrimination observed were so close to 1 that, taking into account the degree of purification of Z_2 and the precision of the assay methods, the two peptides might well be identical

Table 5

Paper chromatography of kallidin, Z_1 and Z_2
No differentiation between Z_2 and kallidin

Liquid system N butanol, acetic acid, water (40 + 10 + 50 vol)
Kallidin applied 750 ng
Z_2 applied A quantity corresponding to 640 ng kallidin by rat uterus assays A Z_2 preparation contaminated with Z_1 was used
Kallidin + Z_2 applied 750 + 640 ng in terms of kallidin
Temparature 22–24°C
Chromatogram run for 14 hours
Length of run 29 cm

Fraction number	Recovery %		
	Kallidin	Z_2 (Z_1 contaminated)	Kallidin + Z_2 (Z_1 contaminated)
1	–	–	–
2	–	–	–
3	–	–	–
4	1 1	–	–
5	4 4	–	–
6	2 7	2 0	–
7	–	2 2	2 0
8	–	9 3	13 2
9	Not tested	2 0	3 0
10	Not tested	–	2 8
11	Not tested	1 0	–
12	Not tested	4 0	2 3
13	Not tested	1 0	–
14	Not tested	–	–
15	Not tested	–	–
16	Not tested	–	–
Total	*8 2*	*21 5*	*23 3*

In a previous paper (BRISEID JENSEN, VENNERØD & DYRUD 1964) significant indices of discrimination were obtained for 3 different batches of Z_2 when compared with bradykinin in rat blood pressure/rat uterus assays *For one of the preparations, which gave an especially high index* value, *the index was considerably reduced* when the direct depressor method was replaced by an adrenaline inhibition method Unspecific contaminants in the Z_2 preparation were considered to be the cause of such a high rat blood pressure/rat uterus assay ratio, which was also observed for one of the two other Z_2-batches examined If the known relative activities of kallidin and bradykinin are taken into consideration, the results given in table 3 in this paper for Z_2-kallidin assays with the same *pair of biological preparations* agree well with the reduced index values of the previously recorded Z_2-bradykinin experiments The results accordingly suggest that the new modification of the purification procedure for Z_2 eliminates the impurities that interfere with the blood pressure measurements

II Guinea pig ileum Indices of discrimination of 0 3 and 0 7 have previously been obtained when Z_2 purified by paper chromatography and synthetic bradykinin were compared for their guinea pig ileum and rat uterus effects (BRISEID JENSEN, VENNEROD & DYRUD 1964) The values mentioned agree reasonably well with the data given by STÜRMER & BERDE (1963) for kallidin and bradykinin In this paper, however, the index values from comparative assays of Z_2 purified by another procedure and kallidin were significantly lower than 1 (table 3) This might indicate that Z_2 is not identical with kallidin, it might also suggest that Z_2 is not homogeneous but contains other polypeptides with similar properties besides kallidin or even unspecific contaminants The index values noticed however, did not differ so far from 1 as to rule out the possibility that the Z_2 preparation tested consisted largely of kallidin

B *Chromatography*

The results given in table 4 show that a Z_2 preparation prepared by the method described in this paper will contain no Z_1 (presumably bradykinin) as could be expected from the results of the model experiment referred to in table 2 Table 4 also shows that Z_2 and kallidin moved together on the paper chromatogram.

For the paper chromat[illegible]
seemed appropria[illegible]
table 5 it can be [illegible]
did not run completely parallel on the paper However, the mixed run of the two preparations did not show any lack of homogeneity, suggesting that Z_2 and kallidin may be identical The reason for a longer run of kallidin in the presence of Z_2 may be partly attributable to the presence of unspecific impurities in the Z_2 preparation In other experiments kallidin also ran slightly less far in single runs

As previously observed (BRISEID JENSEN & VENNEROD 1962 b, c) Z_1, which might be identical with bradykinin ran somewhat faster on the chromatogram under the conditions chosen The content of Z_1 in the Z_2 preparation used was over estimated having been calculated as kallidin which has only about 60% of the activity of bradykinin on rat uterus (STURMER & BERDE 1963)

An over all recovery of activity of 20–25% of Z_2 or of Z_2 + bradykinin in the same liquid system has previously been recorded (BRISEID JENSEN & VENNEROD 1962 c) In the experiment recorded in table 5 the [illegible]

[illegible] acetic acid applied to the

chromatogram, was kept at −17° and assayed on rat uterus against standard bradykinin some days later The activity was the usual 60% of that of bradykinin, showing that loss of activity must have taken place during chromatography However, aliquots of the acetic acid kallidin solution, which were evaporated to dryness at 30° under a pressure of 5–10 mm Hg and then further dried at 22–24° and 0 01 mm Hg, also showed a considerable loss of activity Only 6–7% of the original rat uterus stimulating effect could be detected

Fractions 17–29, and for the single kallidin run also fractions 9–16, were not tested in the experiment summarized in table 5 Similar experiments, however, demonstrated that no significant activities could be detected in those fractions

WEBSTER & PIERCE (1963) found that kallidin ("kallidin-10") on incubation with trypsin at 37° was to some extent recovered as bradykinin ("kallidin-9") They identified the polypeptide by paper chromatography in n-butanol, acetic acid and water (60 + 10 + 27) and used kallidin and bradykinin as standards In the work reported here a Z_2 preparation was incubated with trypsin (Nutritional Biochemicals Corporation, 2x Crystalline) and then submitted to paper chromatography About 17 μg

Table 6

Paper chromatography of kallidin and Z_2
The effect of trypsin on Z_2

Liquid system N butanol acetic acid, water (40 + 10 + 50 vol)
[illegible] by rat uterus assay
[illegible] n terms of kallidin

Chromatogram run for 14 hours
Length of run 27 cm
The figures in parentheses refer to a simultaneous paper chromatogram

Fraction number	Recovery %		
	Trypsin treated Z_2	Z_2	Kallidin + trypsin treated Z_2
1	1 0	– (2 0)	– (–)
2	1 0	3 5 (5 5)	– (–)
3	–	4 0 (1 0)	– (–)
4	–	1 0 (1 0)	– (–)
5	–	1 0 (–)	– (–)
6	– (–)	2 5 (–)	– (–)
7	4 0 (25 5)	–	1 0 (11 0)
8	21 5 (8 5)	–	39 5 (30 5)
9	– (–)	–	3 5 (1 0)
10	–	–	1 0
11–14	–	–	–
Total	27 5	12 0	45 0

of Z_2 was treated with 200 µg of the enzyme, the procedure was that described by DINIZ & CARVALHO (1963) for the release of bradykinin from denatured plasma Table 6 shows that about 12% (10% in a parallel run) of the activity of the Z_2 not submitted to trypsin was recovered in fractions 2–6, and about 26% (34% in a parallel run) of the activity of enzyme treated polypeptide was recovered in fractions 7 and 8 Bradykinin has previously been found to give considerably better recoveries from paper chromatograms than did kallidin in the experiments of the work reported here (see above)

In another run of the chromatogram, shown in table 6, trypsin treated Z_2 was applied together with synthetic kallidin The table shows that no kallidin activity was recovered and that 45% of the total activity (41% in a parallel run) could be detected in fractions 7–10

The results of the trypsin incubation experiment suggest that Z_2 under this treatment will be partly transformed to bradykinin or a substance running like bradykinin in the solvent system used

Discussion

The purification procedure described for Z_2 seems to give a highly purified polypeptide No Z_1 could be detected in paper chromatographic runs even when large amounts of Z_2 were submitted to chromatography in a solvent system known to differentiate between the two substances

Neither the pharmacological parallel assays nor the paper chromatographic experiments differentiated clearly between the concentrates of Z_2 and synthetic kallidin The indices of discrimination from the rat blood pressure/rat uterus assays did not deviate more from 1 than might be expected, and the activity peaks of the two kinins did not separate under chromatographic conditions that would differentiate between Z_2 and bradykinin Further, a trypsin incubation experiment yielded a substance that ran somewhat faster than kallidin and accordingly parallel with bradykinin in the solvent system used

The guinea pig ileum/rat uterus experiments, however, gave indices of discrimination lower than 1, thus suggesting non identity between Z_2 and kallidin However, the deviations were not too large to have been caused by minor quantities of contaminating substances If the low index values are caused by impurities present in significant quantities, these must be polypeptides with properties rather similar to those of *kallidin*

Although no definitive conclusion can be based on the pharmacological and chromatographic experiments carried out, and should preferably be based on the chemical isolation and identification of Z_2, we hold that

the results justify the assumption that kallidin and the main constituent of the Z_2 fraction of the urinary kinins are identical

Summary

A method has been described for preparing a concentrate of a second ary kinin from human urine (Z_2)

A comparison was made between synthetic kallidin (Sandoz) and concentrates of Z_2 Pharmacological parallel assays by rat uterus stimulation and rat blood pressure measurements did not differentiate be tween the substances In paper chromatographic runs Z_2 and kallidin could not be separated under conditions that separated Z_2 and brady kinin By trypsin incubation of Z_2 a substance was formed with chromato graphic properties different from those of kallidin and similar to those *of bradykinin in the solvent system used*

Pharmacological parallel assays by rat uterus and guinea pig ileum stimulation gave discriminating indices differing significantly from 1 The deviations, however, were not too large to have been caused by minor impurities or by polypeptides with properties similar to those of kallidin

The results give support to the view that kallidin and the main con stituent of the Z_2 fraction of the urinary kinins are identical

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REFERENCES

Diniz C R & I F Carvalho A micromethod for determination of brady kininogen under several conditions *Ann N Y Acad Sci* 1963 104 77–89

Jensen K Briseid & A M Vennerød Purification of the main kinin of [illegible] 19 265 274

Jens[illegible] al and chromato [illegible] with bradykinin

Jensen [illegible] al and chromato graphic differentiation between a secondary kinin from human urine and bradykinin *Acta pharmacol et toxicol* 1962 c 19 337 344

Jensen K Briseid A M Vennerød & O K Dyrud Pharmacological properties of human urinary kinins *Acta pharmacol et toxicol* 1964 20 322–328

Pless J E Sturmer, St Guttman & R A Boissonnas Kallidin Synthese und Eigenschaften *Helv chim acta* 1962 45 394–396

Stürmer E & B Berde A pharmacological comparison between synthetic bradykinin and synthetic kallidin *J Pharmacol Exper Therap* 1963 139 38–41

Webster M E & J V Pierce The nature of the kallidins released from human plasma by kallikreins and other enzymes *Ann N Y Acad Sci* 1963 104 91–107

Acta pharmacol et toxicol 1963, 20, 339–350

From the Institute of Medical Chemistry, Uppsala University
Uppsala, Sweden

The Retarding Influence of Atropine on Absorption of Radioactive Diisopropylphosphofluoridate ($DF^{32}P$) and Radioactive Inorganic Phosphate ($^{32}P_i$) in the Rat

By

B. V. Ramachandran[1]) and G. Ågren

(Received December 2 1963)

The most important adjuvant in the oxime therapy of severe organo- phosphate poisoning is atropine, but its potentiating effect is not clearly understood We have been interested in the esterases of the rat liver tha bind large amounts of radioactive diisopropylphosphorofluoridat ($DF^{32}P$) (RAMACHANDRAN & ÅGREN 1963a) While studying the possibl influence of atropine on the incorporation of $DF^{32}P$ in these enzym systems, a chance observation was made that in a rat having receive atropine prophylactically and $DF^{32}P$ subsequently, injected by the sam route the radioactivity taken up in the liver was considerably reduce (ÅGREN & RAMACHANDRAN 1964) The phenomenon has now been foun to be due to a local effect of atropine in retarding the absorption of sul stances administered subcutaneously or intramuscularly The preser paper gives the details A preliminary communication has been publishe (RAMACHANDRAN & ÅGREN 1963b)

Materials and Methods

$DF^{32}P$

England

in 25 ml

were fre[illegible] by diluting the stock solution approximately 25 fold w physiological saline so that 1 ml contained the equivalent of 0 32 mg of $DF^{32}P$ T total radioactivity injected into each rat ranged *from 2 to 6* $\times 10^6$ counts per min under the conditions of our counting and depending on the extent of decay of ³ There was little hydrolytic deterioration of $DF^{32}P$ Carrierfree radioactive inorga phosphate ($^{32}P_i$) was also obtained from the same source Atropine sulphate

[1]) Visiting Lecturer from the National Chemical Laboratory Poona, India.

obtained locally All references to atropine in the text are to the salt Unless otherwise stated the general conditions were as follows Atropine was administered at 17 4 mg/kg (50 μmol) to the rats The volume injected each time was 1 ml When a mixture of $DF^{32}P$ and atropine was to be administered, the necessary amount of atropine was dissolved in the required volume of diluted $DF^{32}P$ solution, another portion of the same solution being used for the control animals When atropine and $DF^{32}P$ were injected separately, the volume of each injection was 1 ml The control rats received 1 ml of saline instead of atropine

Tropine and DL-tropic acid were obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio, U S A These were administered separately or as a mixture at a concentration of 50 μmol of each component/kg The hydrolytic products of atropine were prepared by refluxing atropine with dilute sodium hydroxide to give complete hydrolysis, as determined by the method of ZVIRBLIS *et al* (1956) The hydrolysate was neutralized with hydrochloric acid and diluted with isotonic saline to the required concentration Female albino rats of the Sprague Dawley strain weighing 220–250 g, were used in most experiments The subcutaneous and intramuscular injections were usually given in the thigh, the latter posteriorly to the hip joint After the necessary lapse of time, the rats were killed by a blow on the head and bleeding The liver was removed and homogenized with 0 25 M sucrose and the volume was made up to 100 ml One ml portions were plated in triplicate in glass cups with 0 1 ml 1 N NaOH for radioactivity determinations For the analysis of the tissue at the site of injection, the whole thigh was excised, and the muscular tissue weighing about 7 to 10 g, was dissected from the skin and bones It was weighed and digested with 50 ml of hot 5 N NaOH neutralized with acetic acid and made up to 100 ml Radioactivity was determined in a Tracerlab SC-18 Superscaler with a TGC 2 end window Geiger tube Other details are given where appropriate

Results

The effect of a mixture of $DF^{32}P$ and atropine

When two groups of rats, A and B, were given subcutaneously a mixture of $DF^{32}P$ and atropine and $DF^{32}P$, respectively, and a rat from each group was killed at various intervals, the amounts of radioactivity incorporated in the livers of group A rats were invariably less than those in group B (table 1) Whereas in the control group the activity attained a steady state at 1 hr and did not show any further appreciable increase, in group A there was a progressive increase with time up to 4 hrs Both the groups showed a decline after 12 hrs , but the activities in the livers of group A were throughout less than those in group B up to end the of the observations, viz , 48 hrs

The activities in the muscular tissues at the sites of injection were found to be much higher in group A than in group B This showed that the incorporation of atropine with $DF^{32}P$ resulted in reduced absorption at the site of injection The difference in the local concentration of activity between the two groups became progressively narrower, though even at 48 hrs the group A rat had a detectably higher concentration of activity.

Table 1

Incorporation of radioactive phosphorus in the liver and localization of activity at the site of injection when DF[32]P is injected into rats either alone or with atropine

Time of sacrifice after injection (hrs)	Radioactivity in counts per minute $\times 10^{-3}$				
	In whole liver			In 1 g of thigh tissue	
	Group A	Group B	A as % of B	Group A	Group B
Subcutaneous					
0.5	197	639	30.8	36.4	16.8
1	328	1097	29.9	42.7	8.0
2	283	767	36.9	23.3	6.1
3	590	1075	54.9	63.5	4.9
4	668	1079	61.9	10.8	7.0
6	633	880	71.9	11.1	7.9
12	648	1018	63.7	7.1	6.8
18	565	962	58.9	9.6	4.9
24	409	740	55.3	5.9	4.3
36	248	711	34.9	5.2	4.4
48	262	458	57.2	4.8	3.5
Intramuscular					
0.5	198	660	30.0	175.8	88.5
1	336	1016	33.1	164.6	27.0
1.5	322	863	37.3	187.8	20.0
2	556	800	69.5	83.7	15.4
3	509	823	61.8	89.7	12.8
4	653	812	80.4	119.3	11.2

[illegible] ers not reported

Similar results were obtained when the injections were given by the intramuscular route

The effect of administering atropine before DF[32]P

Table 2 gives the results of administering atropine separately before DF[32]P at various intervals The pairs of injections, each of 1 ml, were given either subcutaneously or intramuscularly in the thigh the control rats were given saline instead of atropine It was observed that here also the uptake of DF[32]P activity in the liver was much lower in the atropinised animals than in the controls There was a localization of activity at the site of injection, but the values showed larger deviations than when DF[32]P and atropine were given as a single injection As will be shown later, atropine seems to have a local effect, and the differences could be

Table 2

The effect of administering atropine at various intervals before $DF^{32}P$ on incorporation of radioactive P into the liver and the localization of activity at the site of injection

Interval between atropine and $DF^{32}P$ (hrs)	Radioactivity in counts per minute × 10^{-3}				
	In whole liver			In 1 g of thigh tissue	
	Group A	Group B	A as % of B	Group A	Group B
Subcutaneous					
0 5	337	737	45 7	170 0	108 4
1	811	1064	76 2	39 6	43 9
2	720	1217	59 2	114 1	21 6
3	729	1140	63 9	49 7	18 0
4	822	1022	80 4	149 6	14 1
Intramuscular					
0 5	198	660	30 0	175 8	88 5
1 0	336	1016	33 1	164 6	27 0
1 5	322	863	37 3	187 8	20 0
2	556	800	69 5	83 7	15 4
3	509	823	61 8	89 7	12 8
4	653	812	80 4	119 3	11 2

Group A rats were given 1 ml of atropine solution subcutaneously or intramuscularly in the left thigh Group B rats received 1 ml of saline After the necessary interval 1 ml of $DF^{32}P$ was injected subcutaneously or intramuscularly in the same thigh The animals were killed 1 hr after the administration of $DF^{32}P$ The radioactivity injected to each rat was 5 7 × 10^6 cpm Each figure represents one rat the whole series is representative of two others not reported

explained on the basis of how close to each other in position the injections had been administered Though the animals invariably were killed 1 hr after $DF^{32}P$, there was an increase with time in the local concentration of activity at the site of injection in the control rats as the time between administration of saline and $DF^{32}P$ decreased This was predictable, since a larger volume derived from two injections has to be cleared as the interval between them is shortened The finding is in accordance with those of WARNER *et al* (1953) for the clearance of radiosodium and SCHRIFTMAN & KONDRITZER (1957) for atropine They observed that, when the injection volume was increased, the rate of clearance was found to decrease for a fixed amount of substance

Administration of atropine 1 hr after $DF^{32}P$ at the same or a different site had no effect on the uptake of $DF^{32}P$ at short or prolonged intervals

Influence of dose level of atropine

The effect of injecting $DF^{32}P$ together with atropine at various levels is indicated in table 3 It was found that the optimum was between 15 and 20 mg/kg, above which there was no additional advantage This range is consistent with the dose level of 17 4 mg/kg used by many workers for

Table 3

The effect of atropine at various levels on incorporation of $DF^{32}P$ in the rat liver

Level of atropine mg/kg	Radio-activity in the liver cpm × 10 ³	Activity as % of control
50	135	25 8
40	258	49 2
30	68	13 0
20	178	34 0
15	145	27 7
10	218	41 6
5	312	59 5
2 5	428	81 7
1 0	476	90 8
none	524	100 0

One ml of $DF^{32}P$ solutions containing the necessary amount of atropine was injected subcutaneously into

series as a whole was repeated once, and several individual experiments were run at various dose levels

protecting rats against lethal organophosphate poisoning (ASKEW 1957; DAVIES *et al* 1959, BERRY *et al* 1959) Atropine was administered by us at this level

Below 15 mg/kg the effect progressively decreased with the dose, but even at the level of 1 mg/kg there was a perceptible retardation of $DF^{32}P$ uptake by the liver

Effect on other organs

To determine whether the presence of atropine in $DF^{32}P$ affected the incorporation in other organs, one rat was injected with the $DF^{32}P$-atropine mixture and one with $DF^{32}P$ subcutaneously at the back of the neck They were killed after 1 hr , and the organs were analysed for radioactivity From the results (table 4) it was observed that there was a uniformly reduced activity in all the tissues analysed, confirming that atropine created a local depot of $DF^{32}P$ at the site of injection and retarded absorption by the system as a whole

Excretion of labelled phosphorus

From the results in table 1 it is seen that the amount of $DF^{32}P$ in the liver of group A decreased after 12 hrs The activity at the site of injection

Table 4

The effect of atropine on incorporation of $DF^{32}P$ in various organs

Material	Radioactivity in cpm in 1 g of tissue	
	Rat A	Rat B
Kidney	48 560	92 750
Liver	16,000	43 000
Lungs	20 700	29,720
Blood (per ml)	8 250	19 000
Pancreas	6 250	9,735
Heart	4,445	7,290
Brain	1,840	3,235
Thigh muscle	800	1,950

Rats A and B, both male, weighed respectively 520 and 450 g Rat A was given $DF^{32}P$ + atropine at the usual dose, and rat B received $DF^{32}P$ alone The amount of *activity injected was 4 4 × 10⁶ cpm The animals were* killed after 1 hr Soft tissue was homogenized with sucrose, and muscular tissue was solubilized with hot alkali

also decreased in group A, almost, though not completely, to the level of group B Since the muscular tissue at the site of injection was not more than about 10 g, the local excess of activity could not account for the

Table 5

Cumulativ excretion of radioactivity by rats when $DF^{32}P$ is injected alone or along with atropine

Excretion *up to* (hrs)	Radioactivity in urine + faeces × 10 ³			
	Group A		Group B	
	Total activity	Activity as % of amount injected	Total activity	Activity as % of amount injected
4	2083	18 1	2558	22 2
8	3633	31 6	3875	33 7
12	5084	44 2	4175	36 3
24	6429	55 9	5939	51 6
36	6939	60 3	6620	57 6
48	7465	64 9	7159	62 3

The gro ps consisted of two rats each These were the same as those used for the 48 hr s were given normal diet and free access to water low and only a few per cent of the total As it was lues were added to those of the latter The urine was diluted wit portions were plated with 0 1 ml of 1 N NaOH *Faeces were ground with 1 N alkali,* boiled and filtered and measured portion were taken *from the neutralized filtrate* The total activity injected to each group was 11 5 × 10^6 cpm

entire deficit in the liver and other organs It therefore seemed of interest to study the excretion of radioactivity when DF³²P was injected with and without atropine Two groups, each of two rats, were given DF³²P + atropine or DF³²P subcutaneously The rats were the same as those used for the 48 hr studies summarised in table 1 It was observed that the group receiving added atropine excreted slightly more radioactivity during the period of observation, 48 hrs There was an initial reduced excretion in group A, presumably due to the well known effect of atropine in interfering with urination The activity excreted in the faeces was only a few per cent of that excreted in the urine and therefore added to the latter value A possible explanation for the increased excretion of radioactivity by group B will be discussed later

Effect of atropine on the absorption of radioactive inorganic phosphate

To investigate whether the effect of atropine is specific to DF³²P or whether the rate of absorption of other substances is also retarded by atropine, carrier free radioactive inorganic phosphate was administered to rats with and without atropine The results are given in table 6 It was found that the rate of uptake of radioactive phosphorus in the liver was much slower when it was injected with atropine There was a considerable local concentration of radioactivity at the site of injection, but this could be demonstrated only when the level of atropine was 50 mg/kg At the lower level the differences were negligible, and the large amount of

Table 6

Effect of atropine on the absorption of labelled inorganic phosphate

$^{32}P_i$ injected (cpm × 10^{6})	Level of atropine (mg/kg)	Time of sacrifice (min)	Radioactivity in cpm × 10^{3}			
			In liver		In 1 g of thigh tissue	
			Group A	Group B	Group A	Group B
Subcutaneous 2 75	50	30	17	182	158	45
	–	60	59	258	109	6
	–	120	100	312	112	9
	17 4	60	53	153	5	3
Intramuscular 5 5	17 4	60	224	427		

Carrier free radioactive inorganic phosphate solution was adjusted to pH 7 0 with 0 01 N NaOH and diluted with physiolo
15 µC under the cond
intramuscular injec
$^{32}P_i$ solution for gr
experimental errors

Table 7

The effect of the hydrolytic products of atropine on $DF^{32}P$ incorporation

Adjuvant to $DF^{32}P$	Radioactivity in the liver cpm $\times 10^{-3}$
Tropine	274
DL tropic acid	307
Tropine + tropic acid	293
Hydrolysed atropine	306
Atropine	87
None (average of 4)	288

The solution of $DF^{32}P$ contained the substances at 50 µ moles/kg of body wt of rat In the tropine + tropic acid mixture each component was present at 50 µ moles/kg Atropine was at the usual level The radio activity injected into each rat was 1 9 × 10^6 cpm The rats were killed 1 hr after the injections which were subcutaneously administered in the flank Each value represents the average of two rats Several experiments were carried out with hydrolysed atropine

activity injected in the intramuscular studies prevented the accurate determination of radioactivity in the tissue

The effect of the hydrolytic products of atropine

Experiments were carried out with tropine, DL tropic acid, tropine and tropic acid and hydrolysed atropine, to determine whether or not any of these had an effect on the absorption rate of $DF^{32}P$ The level at which these were administered together with $DF^{32}P$ was 50 µmoles/kg The mixture of tropine and tropic acid contained each component at this level In no instance was any reduction observed in the activity incorporated in the liver (table 7)

The effect of injecting atropine and $DF^{32}P$ by alternate routes and sites

In several experiments atropine and $DF^{32}P$ were injected by alternate routes In no instance was any reduced uptake of radioactivity by the liver conclusively demonstrable The routes used were pairs chosen from intraperitoneal, intramuscular, subcutaneous and, in a few experiments intravenous The interval between administering atropine and $DF^{32}P$ and between the latter and killing the animal were varied from 5 minutes to several hours The dose level of atropine was also increased up to 100 mg/kg Even when both were subcutaneous, the pairs of injections were

given at sites away from each other The combinations were at any two of the different sites the back at the neck, middle back, lower back, right flank, left flank and loose skin in the abdomen No reduction in the uptake of radioactivity in the liver was observed in any of the experiments Sinec in our experience even under similar conditions the uptake of $DF^{32}P$ can show a deviation of 10% or more from the mean, a large number of experimentswill be needed for assessing the systemic effects, if any, of atropine in retarding absorption At present all that can be concluded is that the effect of atropine is local and observable only if the $DF^{32}P$, which should be injected subsequently, also follows the same route in the tissue through which atropine has passed previously

An other observation made in this connection was that when atropine was injected intramuscularly in the thigh, with $DF^{32}P$ subcutaneously after 1 hr in the same thigh, the uptake of activity in the liver was consistently lower, to the extent of about 80% If, on the other hand, atropine was injected subcutaneously in the thigh, with $DF^{32}P$ intramuscularly after 1 hr in the same thigh there was no reduction in uptake of $DF^{32}P$ by the liver This points to significant differences in the clearance of atropine given by different routes The findings of TØNNESEN (1950) on the rate of excretion of subcutaneously and intravenously injected atropine by rats may be of relevance here

Discussion

The results reported above point to some local effect of atropine in retarding the rate of absorption of substances into the system Even massive doses of atropine injected by alternate routes failed to produce any reduced absorption It has also been found that atropine, *in vitro*, has no inhibitory effect on the binding of $DF^{32}P$ to liver esterases (unpublished results) The reduced uptake of labelled phosphorus by the liver and other organs thus reflects the net result of events taking place elsewhere, and this is evidently at the site of injection, where a higher concentration of radioactivity is found when $DF^{32}P$ is given along with atropine That the effect is not specific to $DF^{32}P$ is indicated by the finding that even labelled inorganic phosphate is poorly absorbed in the presence of atropine SCHOU (personal communication) has confirmed our experiments by observing that the presence of 1 5% atropine in sulphacetamide injected into mice resulted in reduced absorption of the drug

From the present information available it is not possible to offer an explanation for the atropine induced reduction in absorption rate It is unlikely that purely physical factors and "salt effects" play a part, since

tropine, tropic acid or the hydrolytic products of atropine at an equivalent concentration in physiological saline have no effect on the absorption rate (table 7) The problem is inextricably linked to the question of the mechanism of capillary absorption, which is itself far from clear (SCHOU 1961)

There are certain lines along which one might speculate It is likely that atropine is not so rapidly absorbed as the conventional methods of analysis indicate The technique of choice would be to record the clearance of the injected drug from the local area (SCHOU 1961) This has been attempted only by SCHRIFTMAN & KONDRITZER (1957), though they only carried out experiments over short intervals It was found by them that for a given volume of injected solution, the higher the concentration of atropine the slower the rate of absorption Our findings here are in agreement with theirs GOSSELIN *et al* (1955) found that of the ^{14}C-labelled atropine injected into rats, 45 to 50% remained unexcreted even after 48 hours Since, according to the findings of TØNNESEN (1956) and OROSZLAN & MAENGWYN-DAVIES (1962), atropine can bind itself to proteins and amino acids, these results indicate that a substantial part of the injected atropine may be bound at the site of injection to the proteins of the absorbing membrane, thereby reducing the rate of passage of substances across the capillary wall

Secondly, atropine itself may pass through rapidly, but may act on the vascular muscles during its passage, giving reduced blood perfusion in the absorption area This is quite likely, since the retarding effect of atropine is perceptible even several hours after injection, resembling the ocular effects of the drug

Finally, atropine may liberate histamine or 5-hydroxytryptamine, resulting in the phenomenon of "self-depression" observed in the rat (SCHOU 1958)

Besides decreasing the absorption rate, atropine also brings about an ultimate reduction in the total amount of $DF^{32}P$ bound to tissues This may be the result of the formation of a depot of $DF^{32}P$ at the site of injection where it is not protein bound (JANDORF & MCNAMARA 1950) It may then get released slowly into the blood stream, thereby allowing more time for the detoxifying enzyme, DFPase (MAZUR 1946) to hydrolyse it to the innocuous diisopropylphosphate, which does not bind to proteins (JANDORF & MCNAMARA 1950)

Summary

When radioactive diisopropylphosphorofluoridate ($DF^{32}P$) is mixed with atropine and injected into rats subcutaneously or intramuscularly,

the rate and the amount of labelled phosphorus found in the liver and other organs are considerably lower than when $DF^{32}P$ is injected alone There is a local concentration of radioactivity at the site of injection, indicating that atropine retards the absorption of $DF^{32}P$ into the system The same phenomenon is observed even if the atropine is injected a considerable time before the $DF^{32}P$, provided the latter is also administered at approximately the same site in the animal The effect seems to be local and not systemic, as it cannot be demonstrated by giving the injections at different sites or by alternate routes

The amount of radioactivity incorporated in the tissues does not reach the level of the controls even at 48 hours, and the cumulative excretion of radioactivity is slightly higher in the atropinised animals

The absorption of radioactive inorganic phosphate is also retarded by atropine The effect thus seems to be general and not specific to $DF^{32}P$. The hydrolytic products of atropine do not have any effect on absorption

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REFERENCES

[illegible]

Berry W K D R Davies & A L Green Oximes of ωω-di quaternary alkane salts as antidotes to organophosphate anticholinesterases *Brit J Pharmacol* 1959 14 186–191

Davies, D R A L Green & G L Willey 2 hydroxyiminomethyl N methyl [illegible]

Jandorf B J & P D McNamara Distribution of radiophosphorus in rabbit tissues after injection of phosphorus labelled diisopropyl fluorophosphate *J Pharmacol* 1950 98 77–84

Mazur A An enzyme in animal tissues capable of hydrolyzing the phosphorus fluorine bond of alkylfluorophosphates *J biol Chem* 1946 164 271 289

Oroszlan S I & D D [illegible] aminoacids *Biochim* [illegible]

Ramachandran B V [illegible]

Schou, J The influence of histamine and histamine liberators on subcutaneous absorption of drugs *Acta pharmacol et toxicol* 1958, 15, 43–54

Schou, J Absorption of drugs from subcutaneous connective tissue, *Pharmacol Rev* 1961, 13, 441–464

Schriftmann, H & A A Kondritzer Absorption of atropine from muscle *Amer J Physiol* 1957, 191, 591–594

Tønnesen, M The excretion of atropine and allied alkaloids in urine *Acta pharmacol et toxicol* 1950, 6, 147–164

Tønnesen, M On the adsorption of atropine to the plasma proteins *Acta pharmacol et toxicol* 1956, 12, 247–250

Warner, G F, E L Dobson, N Pace, M E Johnston & C R Finney Studies of human peripheral blood flow The effect of injection volume on the intramuscular radiosodium clearance rate *Circulation*, 1953, 8, 732–734

Zvirblis, P, I Socholitsky & A A Kondritzer The kinetics of the hydrolysis of atropine *J Amer pharm Ass, sci Ed* 1956, 45, 450–454

Acta pharmacol et toxicol 1963, 20, 351–356

From the Biological Department, Ferrosan Ltd , Copenhagen

Triamterene Activity Investigated by the Stop-Flow Technique and *In Vitro* Studies on Carbonic Anhydrase

By

Ole E Nielsen and J Buus Lassen

(Received December 11 1963)

Triamterene by virtue of its natriuretic and potassium-retaining effect acts as an aldosterone antagonist, but in adrenalectomized rats also triamterene preserves its characteristic effect on the renal potassium excretion (HERKEN & SENFT 1961, BABA *et al* 1962) Thus, the mechanism may be a direct interference with the tubular transport of potassium or, to be more exact, an interference with the potassium sodium exchange mechanism distally in the nephron

In the ion concentration curves obtained by the stop flow technique the potassium ion manifests itself by a distally situated concentration peak within the area of secretion More proximally, a concentration minimum is often observed, which is taken to include the site of potassium reabsorption The ammonium ion presents a similar distally situated concentration peak, usually parallel to that for the potassium ion

The object of our investigation was to clarify the effect of triamterene on the stop flow distribution of the two cations, as well as their relative excretion rates during the initial clearance periods and to compare the excretion patterns of paraaminohippuric acid (PAH) and triamterene

The increased bicarbonate excretion to be seen after administration of triamterene is the reason for our *in vitro* studies of the effect of the drug on carbonic anhydrase

Methods

Stop-flow experiments For the stop-flow experiments we used pigs (weighing 10–15 kg) anaesthetised with pentobarbital (mebumal NFN) Urethral catheters were inserted into both renal pelves Each kidney was used for one experiment only Thus, one catheter was clamped in the preliminary control experiment, the other being clamped for the stop-flow experiment after administration of triamterene Before and

after the clamping which lasted 3 min in all the experiments clearance determinations were made of the glomerular filtration rate (inulin) and the renal plasma flow (PAH) in both kidneys in the usual way A 3% sodium chloride solution was infused during the experiments to secure a sufficiently large urine volume and a sufficient concentration of sodium ion in the tubules for potassium sodium exchange At the end of the clamping period 30 consecutive urine samples of 0 5 ml each were collected

Carbonic-anhydrase inhibition The carbonic anhydrase inhibiting activity of triamterene was compared with the action of sulphanilamide, acetazolamide and chlorothiazide by PHILPOT & PHILPOT s technique (1936) The enzyme was prepared from bovine erythrocytes, as described by KEILIN & MANN (1940)

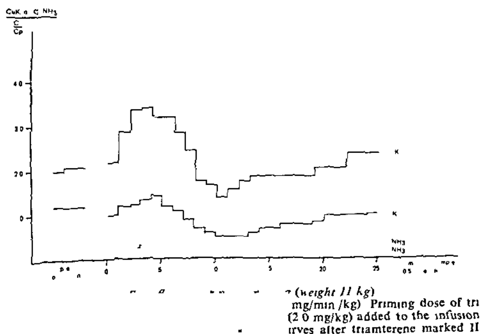
Analytical methods Sodium and potassium were determined by flame photometry ammonia by Conway's microdiffusion method Inulin was determined colorimetrically and triamterene fluorimetrically

Results

Stop flow Experiments

Fig 1 illustrates the stop flow curves for potassium and ammonium ions in the control experiment and after administration of 4 0 mg triamterene per kilogram

The measured concentrations were corrected by the inulin concentration index, but not by the serum ion values, as the serum potassium was constant, and the serum ammonia was not measured The curves, notably the potassium curve, are seen to be displaced to a lower level after administration of triamterene, though on the whole the shape of the curves remained unchanged



(weight 11 kg) mg/min /kg) Priming dose of tri (2 0 mg/kg) added to the infusion irves after triamterene marked II

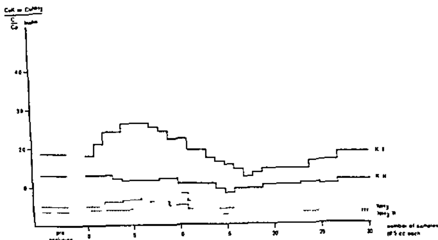


Fig 2 *Stop flow experiment in a pig (weight 15 kg)*
3% NaCl given as continuous i v infusion (rate 0 83 ml/min /kg) Priming dose of triamterene (6 0 mg/kg) given i p, supplementary dose (1 9 mg/kg) added to the infusion Clamping period 3 min Control curves marked I curves after triamterene marked II

Fig 2 shows the result of a similar experiment in which the triamterene dose was 7 9 mg/kg

In this experiment also the levels of potassium and ammonia were lowered after triamterene Unlike fig 1, however, this shows a pronounced change in the shape of the potassium curve, the distal concentration peak having been eliminated The more proximally situated reabsorption minimum seems to have been flattened, but the reality of this alteration is doubtful Similarly, we cannot say with certainty that the concentration curve for the ammonium ion has been altered in shape, although it has become displaced to a lower level

In an experiment with a triamterene dose of 6 0 mg/kg a similar alteration was seen in the stop flow curve for the potassium ion, and an experiment, in which 4 2 mg/kg were given, bore out the observations illustrated in fig 1

In table 1 are recorded the mean values for excreted potassium and ammonium ions during the clearance periods of five stop flow experiments, in which the urine was collected before and after administration of different doses of triamterene Twice the standard deviation is given for each mean value

Although in all these experiments an unmistakeable fall was seen in potassium excretion after triamterene, the ammonium ion showed varying and indeterminate changes

Table 1.

Excretion of potassium and ammonia (mean values ± 2 S D of clearance periods) after administration of different doses of triamterene

Dose mg/kg	Control			Triamterene		
	No of clearance periods	K µeq/min	NH_3 µeq/min	No of clearance periods	K µeq/min	NH_3 µeq/min
4 0	3	82 ± 5 1	26 ± 3 8	6	57 ± 13 4	25 ± 4 6
4 2	3	154 ± 11 0	22 ± 2 0	6	112 ± 17 6	28 ± 7 2
5 8	3	144 ± 2 0	28 ± 4 2	3	90 ± 12 4	40 ± 6 0
6 0	3	95 ± 14 2	36 ± 6 2	3	32 ± 4 2	29 ± 6 4
7 9	3	110 ± 10 4	29 ± 9 8	6	79 ± 4 4	21 ± 3 7

In an earlier investigation on the comparative renal clearances of inulin, PAH and triamterene (BUUS LASSEN & NIELSEN 1964), it was shown that the clearance of triamterene exceeded that of inulin and came close to that of PAH Thus, triamterene is excreted by tubular secretion In some of the present stop-flow experiments the shape of the stop-flow curves for triamterene and PAH were determined, these investigations showed that triamterene has a concentration maximum in the same proximal area as PAH

Carbonic Anhydrase Inhibition In Vitro

In table 2 are listed the results of *in vitro* investigations into the effect of triamterene on carbonic anhydrase, compared with the effects of sulphanilamide, acetazolamide and chlorothiazide Our results on the action of the three last-named drugs have been compared with those of a similar investigation by BEYER (1958)

Table 2

Comparison of three known carbonic anhydrase inhibitors and triamterene

	Molar concentrations required to inhibit the carbonic anhydrase in vitro 50%	
	Present investigation	BEYER (1958)
Sulphanilamide	1 7 10^{5}	1 3 10^{5}
Acetazolamide	7 9 10^{8}	7 2 10^{8}
Chlorothiazide	2 1 10^{6}	1 7 10^{6}
Triamterene	>6 3 10^{-2}	–

The figures which indicate the molar concentrations of the drugs that produce a 50% inhibition of the enzyme show that triamterene has no inhibitory effect on carbonic anhydrase at molar concentrations up to 6.3×10^{-2}

Discussion

The results of these stop flow experiments bear out the assumption that triamterene alters trans tubular potassium transport On administration of about 4 mg of the drug per kilogram body weight the shape of the potassium curve did not change but fell to a lower level This observation was confirmed by SENFT by stop-flow experiments on rats (personal communication (1963)) With a dose of 6–8 mg/kg a flattening of the distal concentration peak was noticed

As pointed out by BERLINER (1959) there is much evidence to suggest that by far the larger proportion of potassium excreted in the urine is secreted by the tubules and that the greater part of the filtered potassium is reabsorbed In our short term experiments no alteration was seen in glomerular filtration rate nor in renal plasma flow as estimated on the basis of inulin and PAH clearance values before and after administration of triamterene The considerable fall in potassium excretion can hardly be attributed to a decreased filtration or an increased reabsorption rate, but is presumably due to depression of the potassium secretion in the distal part of the nephron As stated the shape of the potassium curves was maintained after fairly small doses of triamterene whereas the secretion peak was found to be eliminated after larger doses A contributing cause of this may be the period of clamping the ureter being the same in all the experiments Prolonged clamping in the experiments with the largest doses might have produced the usual concentration peak in the distal part of the curve

Triamterene did not appear to affect the tubular distribution of ammonia as much as that of potassium Previous investigations have shown that triamterene reduces urinary ammonia excretion in human subjects (BUUS LASSEN & NIELSEN 1964) However, the values set out in table I show only small and uncertain alterations and the same is true of the stop flow curves A comparison between the stop flow curves of PAH and [illegible] to increase renal bicarbonate excretion is not due to an inhibition of carbonic anhydrase The same observation was made by BABA (1963)

Summary

In stop-flow experiments on pigs it has been shown that triamterene at doses of 4 mg/kg lowered the concentration level of the potassium ion and that doses of 6–8 mg/kg also eliminated the concentration peak situated distally in the curve. These observations suggest that triamterene depresses tubular potassium secretion. Similar alterations were not demonstrable for the ammonium ion. *In vitro* investigations showed no inhibition of carbonic anhydrase

Acknowledgements.

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REFERENCES

Baba, W J, G R Tudhope & G H Wilson Triamterene, a new diuretic drug *Brit med J* 1962, II, 756–760

Baba, W J 1963, quoted by Wilson, G H *Brit med J* 1963, I 285–292

Berliner, R W Renal mechanisms for potassium excretion *The Harvey Lectures* 1959–60, 55, 141–171

Beyer, K H The mechanism of action of chlorothiazide *Ann New York Acad Sc* 1958, 71, 363–379

Herken, H & G Senft 2-,4-,7-triamino-6 phenyl pteridin als "Aldosteron antagonist" *Klin Wchr* 1961, 39, 1205–1206

Keilin, D & T Mann Carbonic anhydrase Purification and nature of the enzyme *Biochem J* 1940, 34, 1163–1176

Lassen, J B & O E Nielsen Investigations on the diuretic effect and the elimination of triamterene *Acta pharmacol et toxicol* 1964 20, 309–316

Philpot, F J & J St L Philpot A modified colorimetric estimation of carbonic anhydrase *Biochem J* 1936, 30, 2191–2193

Acta pharmacol et toxicol 1963, 20, 357–366

From the Pharmacological Laboratory, A/S Alfred Benzon, Copenhagen Denmark

A Quaternary Atropine Derivative

By

Steen Antonsen and G Hegermann Nielsen

(Received December 16 1963)

Therapeutic use of propionyl atropine methyl nitrate (PAMN[1]) has been undertaken on the basis of experiments reported by HERMAN, SHAW & ROSENBLUM (1958) and HERMAN & SHAW (1958)

The work of these authors included, amongst other things, demonstration of the compound s peripheral anticholinergic action, neuromuscular blocking action, ganglion blocking action and inhibitory action on gastric secretion in Shay rats

We believe to have demonstrated that the compound is even more suitable for use in ulcer therapy than appears from the above mentioned publications since not only we have found a reliable effect in Shay-experiments with much smaller doses, but were also able with this drug to protect rats under stress against stomach injury

We have further, sought to place PAMN in proper relation to atropine on the one hand and on the other hand to the newer synthetic anticholinergics

Experimental methods

Antagonism to acetylcholine and barium chloride on isolated guinea pig ileum
Suitable pieces of ... aerated by ca ... graph are pr ... or barium ch ... with the antagonisti ... chloride We have p ...

[1]) Protrop n ®

reported by SCHILD (1947) for determination of pA_2, according to which the piece of ileum is in constant contact with the antagonist

Anticholinergic and ganglion blocking effect in the cat Cats of 1 5–2 5 kg are anaesthetized by intramuscular injection of 2–2 5 ml of solutio hypnopheni (100 mg allypropymal NFN and 112 mg diemal NFN per ml) The blood pressure is recorded by a mercury manometer connected to the right carotid artery and injections are given through a glass cannula in the femoral vein The distal end of the intersected left sympathetic nerve is stimulated electrically through platinum electrodes, and the contractions of the nictitating membrane are recorded In order to measure the short lived ganglion-blocking effect of the drugs here investigated, they must be administered along with simultaneous constant stimulation of the nerve A suitable dose of acetyl choline (about 4 µg) is given at regular intervals, and the influence of the anticholinergic drugs on the resultant depressor effect is measured

Neuromuscular blocking action on the isolated rat diaphragm The method reported by BÜLBRING (1946) *is used A piece of rat diaphragm with its right phrenic nerve is* hung in oxygenated Tyrode solution (25°) Muscle contractions are produced by electric stimulation of the nerve, when they have become constant, the test material is introduced into the bath and left there for three minutes before changing the Tyrode fluid The percentage reduction in the height of the curve during this period is used as a measure of the blocking action

Shay rats The procedure is substantially as previously reported (ANTONSEN 1953) Female rats weighing about 170 g are placed individually in wide mesh wire cages 24 hours before the operation The animals receive no food, but have access to a solu tion containing 5% glucose and 0 4% sodium chloride After a ligature has been placed around the pyloric sphincter under light ether anaesthesia, and the operation wound has been sutured, either a test dose or 1 ml 0 9% sodium chloride solution is given intraperitoneally, and the animals are returned to their cages, but now without access to the solution They remain there for eight or sixteen hours before they are chloroformed The stomach is removed, and cut open along the greater curvature and the contents are poured into a graduated centrifuge glass After centrifuging the fluid is potentiometrically titrated, first to pH 3 5 and then to pH 9 2 The opened stomach is spread out with pins and examined for ulcers at a tenfold magnification The degree of ulceration is estimated as earlier described (ANTONSEN 1953)

Stress rats Female rats weighing about 190 g are placed in wide mesh wire cages for 24 hours without food, but with access to a solution containing 5% glucose and 0 4% sodium chloride The test dose or 1 ml 0 9% sodium chloride solution is ad ministered intraperitoneally 15 min before the animals under light ether anaesthesia are placed in the immobilization cages described by LAMBLING & BONFILS (1960) The cages are 20 cm long tubes of 5 cm diameter, made of strong wire mesh The legs of the animals are drawn through four holes in the bottom of the tube and tied to gether in pairs with adhesive tape, so that the animals can only move slightly The cages remain fastened in stands about 50 cm above the floor for an experimental period of eight or sixteen hours After the animals have been chloroformed, their stomachs are removed, cut open along the lesser curvature, spread out with pins and then examined at 15 × magnification The ulcers in these animals are formed exclu sively on the glandular portion of the stomach and mainly near the greater curvature The degree of ulceration is here estimated according to the scale 0 normal stomach 1 clear hemorrhage, 2 a few small ulcers, 3 numerous small ulcers or a single large one

Mydriatic activity The pupil dilating effect of the test compounds is demonstrated

in female mice weighing 20–25 g after intravenous injection as well as oral administra-

Toxicity The LD50 was determined in female mice after intravenous, intraperitoneal or subcutaneous injection or oral administration to animals that had been fasted from the previous evening

Results

Action on Isolated Guinea Pig Ileum

A direct comparison between PAMN and atropine, based upon experiments in which these compounds were administered 120 sec before acetylcholine, was not possible In seventeen tests we found for the activity ratio PAMN atropine, values ranging from 1·2 8 to 1200 1 In fig 1 is shown a series of dose response curves found in this way; although it is clearly evident that PAMN shows a greater potency than does atropine, a quantitative comparison cannot be made In experiments done by the method of SCHILD (1947), the pA_2 for PAMN was found to be 10 36, and for atropine 9 06, which means that in these circumstances PAMN is about 35 times as potent as atropine With barium chloride as a stimulant it was likewise impossible to make a direct comparison of the two drugs' action on the same piece of ileum, further, in such experiments a sufficient

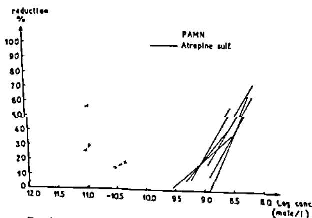


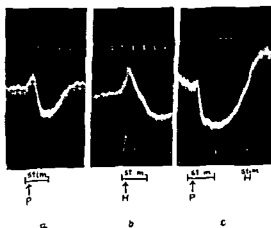
Fig 1 Anti acetylcholine activity on the guinea pig ileum

number of similar curves could not be obtained for determining the pA_2-values with reasonable accuracy However, it could be shown that PAMN has an activity about seven times as great as that of β (diphenyl methoxy) ethyl-trimethyl-ammonium iodide (metropin ®), which itself is about fifteen times as potent as papaverine A series of comparative experiments confirmed that PAMN has as high a spasmolytic effect as about 50–75 times that of papaverine

Effect on anaesthetized cats

In doses of 0 1–0 5 mg PAMN produced a transient fall in blood pressure, the depressor effect produced by acetylcholine was inhibited for 10–60 minutes At a dose of 0 375 mg, PAMN administered during continuous stimulation of the sympathetic nerve reduced the contraction of the nictitating membrane by about 50%, but the action was extremely short (see fig 2) Administration of the same amount of hexamethonium *resulted in about the same reduction in contraction,* but the action persisted for 15–30 minutes Atropine (0 75 mg) inhibited the depressor effect of acetylcholine for a shorter period than did PAMN (0 375 mg), and it exerted only an extremely slight, transient ganglionic blocking action

The neuromuscular blocking action on the rat diaphragm is indicated in table 1 Although PAMN, as expected, had a significantly greater potency than atropine, its action came only to about 50% that of metropin and 2% that of D tubocurarine chloride, an activity clearly of no significance in oral intake of therapeutic doses of the drug



[illegible] 0 5 mg

[illegible]

Table 1

Neuromuscular blocking action

Compound	Dose mg	Reduction %
D Tubocurarinchl	0 08	6
-	0 12	23
PAMN	4 0	9
-	5 6	23
-	8 0	51
METROPIN ®	2 0	5
-	3 0	31
	4 0	47
Atropine sulph	8 0	0
-	16 0	21

In *Shay rat experiments* PAMN has both an inhibitory action on the stomach secretion and a protective action against ulcer formation

The results of two tests are shown in table 2 Metropin (2 5 mg) can, as shown by earlier investigations (ANTONSEN 1953), protect Shay rats fairly well for 8 hours, and the same degree of action was obtained with 0 1 mg PAMN In 16 hour experiments 0 5 mg PAMN had a considerable effect, in the animals receiving this dose no real ulcer formation occurred, only irritation and hemorrhage In contrast, a ten times larger dose of atropine – a dose that caused almost maximal pupil dilation during the entire experimental period – could not reduce the quantity or the acidity of the secretion or protect against ulcer formation

Action in immobilized rats is summarized in table 3 A distinctly protective effect of 0 5 mg PAMN was seen, involving a significant reduction in the occurrence of pathological changes in the stomach,

Table 3

Stress-experiments on rats test period 16 hours

Compound	Dose	Number of animals	Ulcer index
0 9 % NaCl	1 ml	18	1 89 × 83 % = 157
PAMN	0 5 mg	18	0 89 × 33 % = 30
Atropine sulph	5 0 mg	16	2 00 × 87 % = 175

Table 2.

Experiments with Shay rats

Compound and dose	Test period, hours	Number of animals	Ulcer index	Gastric contents			
				Volume ml	pH	Titration values meq NaOH	
						Ad pH 3 5	Ad pH 9 2
0 9 % NaCl 1 ml	8	8	1 19 × 87 % = 104	12 3	1 53	0 808	0 974
PAMN 0 10 mg	8	8	0 12 × 25 % = 3	7 2	1 65	0 488	0 624
PAMN 0 50 mg	8	8	0	4 6	2 21	0 182	0 290
Metropin 2 50 mg	8	8	0 12 × 25 % = 3	7 0	1 45	0 504	0 640
0 9 % NaCl 1 ml	16	9	2 39 × 89 % = 212	13 7	1 61	0 426	0 665
PAMN 0 50 mg	16	9	0 67 × 77 % = 52	10 2	2 40	0 159	0 444
Atrop sulph 5 00 mg	16	9	1 72 × 77 % = 133	10 8	1 69	0 399	0 613
Propanthel brom 1 00 mg	16	9	1 19 × 62 % = 74	6 2	2 06	0 216	0 407

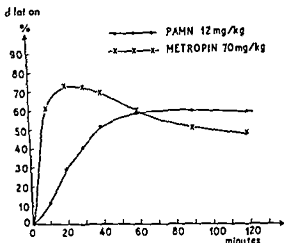


Fig 3 Mydriatic activity in mice Oral administration
Abscissae Time after administration
Ordinates Dilations of pupils as percentage of maximal pupil diameters
(Each curve represents the average response of five animals)

compared to what was found in control animals, whereas a dose of atropine sulphate ten times as large was completely without a protective effect

The mydriatic activity in mice after oral administration of PAMN and metropin is shown in fig 3 The onset of action of PAMN was slower, but the duration of action longer than that of metropin The same fact was noticed after intravenous injection of the two drugs (fig. 4), though 30 minutes after injection there remained only 17% of the maximum activity of metropin, there remained 70% of PAMN's activity

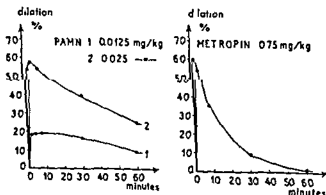


Fig 4 Mydriatic activity in mice I v administration

Table 4

LD50 values for mice mg/kg

Compound	Route of administration			
	i v	i p	s c	oral
PAMN	8 4	80	250	500
Metropin	4 5	66	85	190
Atropine sulph	72	230	>600	450

The acute toxicity in mice is seen from table 4 The LD50 for intraperitoneal and intravenous administration was about the same for PAMN and metropin After subcutaneous injection PAMN was poorly absorbed, resulting in a high LD50 The compound caused severe necroses at the site of injection The LD50 by oral administration was also high, perhaps because the substance administered in this way enters the central nervous system only to a slight degree When the animals died after oral administration of the drug, death occurred within 10–15 minutes

Discussion

PAMN had undoubtedly a clear anticholinergic action on the isolated guinea-pig ileum The finding by HERMAN, SHAW & ROSENBLUM (1958), that atropine acts 4 times as strongly, was probably due to the doses used having been large PAMN has a very flat dose response curve, which means that at low dosage PAMN still retains some of its pharmacological activity, whereas correspondingly low doses of atropine are completely inactive Another difficulty in these determinations is that the effect of PAMN on the ileum is so prolonged that numerous washings are necessary before one can depend on the action of a new dose of acetylcholine

Likewise a prolonged anticholinergic action was seen on the mouse pupil, where the dilation after administration of PAMN lasted much longer than a similar dilation produced, for example, by metropin

In anaesthetized cats PAMN's anticholinergic action was also of long duration, whereas the ganglion blocking action was extremely short and hardly of any significance for the clinical use of the drug (BAINBRIDGE & BROWN 1960) Just as insignificant, undoubtedly, is the increase in the neuromuscular blocking effect, which has been brought about by the quaternisation of atropine

On the guinea-pig ileum, besides the anticholinergic action, there was

shown considerable spasmolytic action against contractions produced by barium chloride, an effect that must be considered of importance in the clinical use

Just as one would have expected from a strong anticholinergic substance, PAMN exerted a powerful inhibitory action against acid secretion by Shay rats as well as against ulcer formation It is, however, perhaps more noteworthy, that PAMN also had a much stronger protective effect than atropine against stomach ulcer in stress rats, in which impulses of central origin produced by the psychic strain must be considered critical for the pathologic changes in the stomach It has been shown that quaternary atropine compounds pass the blood brain barrier only with great difficulty (HANCE *et al* 1963), making it hard to believe that PAMN exerts its effect through the central nervous system One must assume that PAMN's strongly spasmolytic action peripherally protects the stomach against the effect of the impulses The difference in action from atropine is not due, at any rate not exclusively, to the prolonged action of PAMN, since in 8 hours stress experiments we have also found a more favourable effect of PAMN than of atropine

Summary

The quaternary compound propionyl atropine methyl nitrate (PAMN) has a more powerful anticholinergic action than atropine on the isolated guinea pig ileum and a much more powerful antagonistic action against barium chloride induced spasms than has papaverine A direct quantitative comparison is difficult to make, because PAMN has an unusually flat dose response curve, moreover, its action on the receptors of the organ is protracted

Prolonged anticholinergic action is seen in mydriatic tests on mice and in antagonism to intravenously administered acetylcholine in anaesthetized cats

PAMN has more pronounced ganglion blocking and neuromuscular blocking action than atropine, but neither actions can be considered of importance clinically

Inhibitory effects against acid secretion as well as against ulceration are found in experiments with Shay rats, and in stress rats the stomach is protected by PAMN, but not by atropine

Acknowledgements

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REFERENCES

Antonsen, Steen Comparison of the Atropin like Actions of some Quaternary Ammonium Compounds and their Effects on 'Shay Rats *Acta pharmacol et toxicol* 1953, 9, 47–58

pharmacological Study of Gamma-Aminobutyryl Choline, Gamma-Aminobutyric Acid, Physostigmine and Atropine *J Pharmacol* 1963, 140, 385

Herman, Laura, F H Shaw & E J Rosenblum The Pharmacology of Propionyl Atropine Methyl Nitrate *J Pharm Pharmacol* 1958, 10 348–355

Herman, Laura & F H Shaw The Action of Propionyl Atropine Methyl Nitrate on Gastric Function *J Pharm Pharmacol* 1958, 10, 356–362

Lambling, André & Sergé Bonfils Das Ulkus in der Zwangssituation *Ärztl Forsch* 1960, 14, 208–212

Quinton, R M The Mydriatic Response of Mice to Atropine *J Pharm Pharmacol* 1963, 15, 239–250

Schild H O pA, a New Scale for the Measurement of Drug Antagonism *Brit J Pharmacol* 1947, 2, 189–206

Acta pharmacol et toxicol 1963, 20, 367–370

From the Department of Pharmacology, University of Copenhagen
(Professor K O Moller, M D)

The Fate of Decamethonium in Doubly Nephrectomized Rabbits. Preliminary Results

By

Christian Broen Christensen and Jens Schou

(Received January 11, 1964)

A rapid efflux of decamethonium and other quaternary ammonium compounds from the cerebrospinal fluid has recently been demonstrated (SCHANKER, PROCKOP, SCHOU & SISODIA 1962) The results for decamethonium were obtained by measuring the radioactivity remaining in the cerebrospinal fluid at various times after injecting by a technique described by PROCKOP, SCHANKER & BRODIE (1962) known amounts of ^{14}C-labelled decamethonium into the lateral cerebral ventricle of rabbits

An attempt to account quantitatively for the intraventricular dose of decamethonium in doubly nephrectomized rabbits was made by measuring the concentration of ^{14}C-decamethonium in plasma and cerebrospinal fluid and multiplying these concentrations with the commonly accepted values for volumes of extracellular fluid and cerebrospinal fluid, respectively These calculations, however, were unsuccessful when based on the volume for distribution of methonium compounds commonly accepted, according to the literature (*e g* PATON & ZAIMIS 1952), as being the extracellular water, viz about 20% body weight (KRUHOFFER 1946) The amounts of radioactivity that could be accounted for by these calculations were much lower than the doses injected

In a few experiments known doses of decamethonium-^{14}C were therefore injected intravenously into doubly nephrectomized rabbits The decamethonium space calculated from measurements of the radioactivity in the plasma 6 hours after injection, proved to be about 60–70% body weight in four experiments (SCHOU 1961, unpublished results)

In the experiments reported below the concentration of decamethonium in the plasma of rabbits after intravenous injection was followed further.

Methods

Male albino rabbits of a single strain, weighing from 2 3–3 3 kg, were anaesthetized with N_2O, O_2 and ether *via* a tracheal cannula before double nephrectomy Then 105 µg/kg ^{14}C-labelled decamethonium bromide (The Radiochemical Centre, Amersham, England), with a specific activity of 12 0 µC/mg, were injected intravenously, and blood samples (1 ml each) were withdrawn at frequent intervals during six hours

For measurement of radioactivity, zinc hydroxide precipitation (SOMOGYI 1945) was used to remove protein from plasma after addition of unlabelled decamethonium bromide to the samples After spinning 500 µl clear supernatant from a sample were added to 10 ml of a scintillation medium (BRAY 1960), and the radioactivity was measured with a Packard Tri-carb liquid scintillation spectrometer, model 314 EX The efficiency of the measurements was checked by comparison with plasma samples containing known added amounts of ^{14}C decamethonium bromide carried through the standard procedure These samples showed a linear relation between added radioactivity and count (after subtraction of background) in the area of the experimental samples In all measurements of radioactivity, the count exceeded 5000 and was at least 5 times the background count The measurements of the radioactivity allowed the concentration of decamethonium in the plasma to be calculated The results are expressed as ng/ml plasma

Results

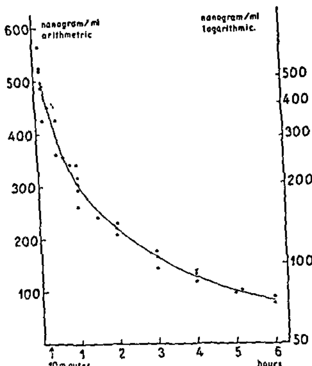
To reduce the total amount of blood sampled from each of the five experimental animals, only 29 samples, obtained at various times after the intravenous injections, were taken for decamethonium analyses The results of these analyses, expressed as ng/ml plasma, are plotted in fig 1 A probable mean curve is drawn through the area of experimental values

This curve has been transformed into a semi-logarithmic system by plotting the logarithms of the plasma concentrations against the time (fig 1, dotted line)

In unpublished experiments on the same strain of rabbits the mean extracellular compartment, expressed as the inulin space, was determined as 20% of the body weight in several experiments A dilution of the injected dose of decamethonium in the plasma similar to the one obtained for inulin at the equilibrium condition is already reached 10 minutes after the intravenous injection Consequently the fate of decamethonium in the body of nephrectomized rabbits cannot be explained by assuming distribution in the extracellular compartment only

Discussion

The results confirm that the fate of decamethonium cannot be accounted for only by distribution in the extracellular water and elimination through the urine After intravenous injection into doubly nephrectomized rabbits an initial rapid decrease in plasma decamethonium concentration occurs



Abscissa time in hours after injection
Ordinate concentration of decamethonium in plasma (ng/ml) Left axis and full line arithmetric plot Right axis and dotted line logarithmic plot

and lasts for about 60 minutes This initial phase gives place to one of slower decrease, linear on a semi logarithmic plot, until the end of the experimental period 6 hours after the intravenous injection Assuming plasma and total extracellular water (including plasma) to be 5 and 20% of the body weight, respectively, only 5 and 20% of the injected dose remained in these compartments at the end of the experiments

The experiments of WASER & LUTHI (1957) showed that decamethonium accumulates specifically in the neuromuscular endplates of mice From these results the initial steep decrease in plasma concentration may well be interpreted as being due to a specific uptake of the labelled decamethonium in the neuromuscular junctions The biological effect of the substance, observed by recording the respiration, lasted only for few minutes after the injection, during the period of the highest plasma concentrations

Decamethonium is considered not to be metabolized in the body

PATON & ZAIMIS (1952) could account for 90% of the injected decamethonium as the parent substance in the urine for 24 hours after the administration to intact animals In our experiments CO_2 was collected from the expired air and did not contain significant amounts of ^{14}C

Methonium compounds are, however, excreted into the bile (cf SCHANKER 1962), and the absorption of decamethonium from the intestine is inconsiderable It therefore seems likely that the slow lasting decrease in concentration of decamethonium in the plasma, which follows a first order function, expressing an elimination mechanism depending on this concentration, is due to excretion of decamethonium in the bile

Further studies of the factors responsible for the two-phased decrease in the plasma concentration of decamethonium after intravenous injection into doubly nephrectomized rabbits are in progress

Summary

The plasma concentration of ^{14}C-decamethonium injected intravenously into doubly nephrectomized rabbits showed a rapid decrease, lasting for about 60 minutes, and then a slow, first-order decrease for the rest of the 6 hour experiments The initial decrease is possibly explained by a specific uptake into the neuromuscular endplates, the lasting slower decrease being assumed due to biliary excretion of decamethonium

Acknowledgements

We are most grateful to Troels Munkner, M D, of the radioisotope laboratory of Rigshospitalet, Copenhagen, for permission to use the Packard Tri-carb instrument of this laboratory Miss Karin Dyhrfjeld gave technical assistance

REFERENCES

Scand 1946 11, 16–30

Paton W D M and Eleanor J Zaimis The methonium compounds *Pharmacol Rev* 1952 4 219–253

Prockop L D L S Schanker and Bernhard B Brodie Passage of lipid insoluble substances from cerebrospinal fluid to blood *J Pharmacol* 1962, 135 266–270

Schanker L S Passage of drugs across body membranes *Pharmacol Rev* 1962 14 501–530

Schanker L S L D Prockop J Schou and P Sisodia Rapid efflux of some quaternary ammonium compounds from cerebrospinal fluid *Life Sciencies* 1962 1 515 521

Som[illegible] M Determination of blood sugar *J Biol Chem* 1945 160 69 73

Was[illegible] von ^{14}C [illegible] hen end

Acta pharmacol et toxicol 1963, 20, 371–374

From the Department of Pharmacology, University of Goteborg Goteborg, Sweden

β-Hydroxylation of Tyramine in Vivo

By

Arvid Carlsson and Bertil Waldeck

(Received January 17 1964)

The mechanism of action of tyramine (TA) is in many ways not clear After sympathetic denervation or cocaine treatment the sympathomimetic actions of TA are markedly reduced (FLECKENSTEIN & BURN 1953, FLECKENSTEIN & STOCKLE 1955) Availability of noradrenaline (NA) in the tissues is essential for its action (CARLSSON *et al* 1957, BURN & RAND 1958, FURCHGOTT *et al* 1963) Displacement of NA from hypothetical storage sites in or at sympathetic nerve endings has been suggested as the basis of its action

Little is known about the fate of administered TA in the living organism. TA is a good substrate for monoamine oxidase (MAO) (BLASCHKO, RICHTER & SCHLOSSMANN 1937, ZELLER 1959), and recently the isolated rabbit heart has been found to form norsynephrine (MUSACCIO & GOLDSTEIN 1963), in this paper called octopamine (OA) To achieve further insight into the mode of action of TA we thought it of interest to pursue investigation of its fate in the intact organism

Material and Methods

an
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ca
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au [illegible] elsewhere detailed report of the technique

1) Tyramine-2 ^{14}C HCl 4 25 mC/mM was obtained from California Corporation for Biochemical Research Los Angeles U S A

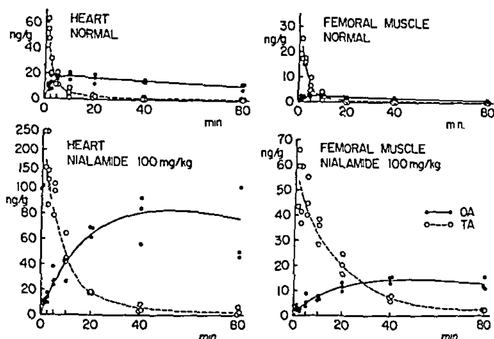


Fig 1 Formation of octopamine (OA) from tyramine (TA) in heart and femoral muscle of mice
Upper graphs ^{14}C TA 0 1 mg/kg intravenously
Lower graphs Nialamide 100 mg/kg intraperitoneally 2 hours before administration of ^{14}C TA 0 1 mg/kg intravenously

Results and Discussion

TA was taken up by heart and femoral muscle (fig 1) It was rapidly converted to OA, which remained in the tissues longer The heart has a higher capacity to form and store OA than has femoral muscle, which may be related to its higher supply of adrenergic nerves

Some experiments were done on rats from which the left superior cervical ganglion had been removed ten days before ^{14}C-TA was recovered in larger amounts in the denervated than in the intact submaxillary gland, indicating the existence of extraneuronal binding sites for TA Significant amounts of OA were found in the intact gland only A notable finding was that 30 min after intravenous administration of ^{14}C-TA to rats pre treated with nialamide, the sums of TA and OA were about equal in both glands These results indicate that the side-chain hydroxylation of TA occurs in the adrenergic nerves

If the mice (fig 1) were pretreated with 100 mg/kg nialamide intraperitoneally 2 hours before administration of ^{14}C-TA, much larger amounts of both TA and OA were recovered, and OA now accumulated at about the same rate as TA disappeared Oxidative deamination and β-hydroxylation thus seem to be the two main metabolic pathways for TA

Table 1

serpine
^{4}C-TA

	Heart		Femoral muscle		
	TA	OA	TA	OA	*n*
C	2 4 (0 2)	17 9 (2 1)	0 5 (0 2)	1 9 (0 5)	4
R	0 0 (0 0)	0 1 (0 1)	0 2 (0 1)	0 1 (0 0)	2
G	1 2 (0 2)	0 1 (0 1)	0 5 (0 1)	0 1 (0 0)	3
N	11 8 (2 3)	68 (13)	11 8 (0 5)	12.0 (0 8)	6
N + R	5 7 (1 1)	14 0 (1 5)	14 3 (2 9)	5 7 (0 3)	2
N + G	10 1 (5 4)	11 9 (1 6)	13 4 (1 8)	4 7 (1 2)	2

Reserpine (10 mg/kg) and guanethidine (50 mg/kg) were given intraperitoneally 6 and 1 hours, respectively, before intravenous administration of ^{14}C-TA After 30 min the animals were killed Reserpine almost completely suppressed the accumulation of TA and OA in the heart In femoral muscle, where possibly a higher proportion of TA is free or unspecifically bound, TA was less reduced Guanethidine showed the same effect on OA, but had a smaller effect on TA uptake When the MAO was inhibited by nialamide (100 mg/kg i p 2 hours before ^{14}C-TA) the effects of reserpine and guanethidine were much less pronounced

The main findings of our investigation may tentatively be explained Accumulation of TA may occur in adrenergic nerves as well as extraneuronally These two locations seem to compete for extracellular TA Under normal conditions intraneuronal accumulation will ultimately prevail Intraneuronally three processes compete for the TA, namely a) oxidative deamination by the MAO of the mitochondria, b) formation of OA by the β-hydroxylase of the storage granules and c) incorporation of TA (and OA) into the storage complex of the granules After preganglionic sympathetic denervation TA will remain on the extraneuronal binding sites and no OA can be formed After reserpine treatment, on the other hand TA is still transferred into the cytoplasm of the nerve endings (which still predominate over the extraneuronal binding sites) but will not be incorporated into the storage complex It will thus remain unprotected against MAO This is true also of any OA formed (Whether reserpine

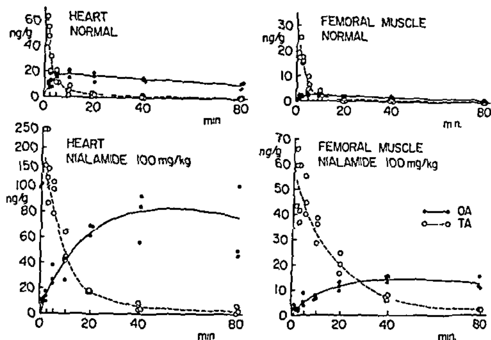


Fig 1 Formation of octopamine (OA)

Upper graphs

Lower graphs lministration of

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Acta pharmacol et toxicol 1963, 20, 375–384

From the Department of Pharmacology, University of Uppsala, Sweden
(Professor Ernst Bárány, M D)

Tubular Excretion of Dopamine (3-hydroxytyramine) by the Chicken Kidney

By

Erland Sanner

(Received November 28 1963)

Urinary excretion of dopamine (3-hydroxytyramine) was first demonstrated by HOLTZ *et al* (1942) and later confirmed by v. EULER *et al* (1951) A detailed study of the excretion mechanism was, however, not carried out Tubular secretion of serotonin (5-hydroxytryptamine) has been demonstrated by SANNER & WORTMAN (1962), the excretion mechanism was studied in more detail by SANNER (1963) Since dopamine has been shown in preliminary studies to reverse a previously reported reserpine-induced blockade of certain diuretic agents (NECHAY & SANNER 1961), it was thought of interest to obtain more detailed information on the mechanism of dopamine excretion

Material and Methods

The general proced[illegible]
W[illegible]
Sp[illegible]

A *Animals*

Rhode Island Red chickens, 2 years old and weighing 1 9–2 8 kg, were used They were kept on commercial chicken food, with fresh water ad libitum. Generally the birds were fasted over night before an experiment. The birds were pre treated with 100 mg/kg of an irreversible monoamine oxidase (MAO)-inhibitor (malamide hydrochloride Niamid ® Pfizer) which was injected into the wing vein about 17 hours before the experiment In one experiment (Expt no 538) 200 mg/kg malamide were given. In another experiment (Expt. no 481) Catron ® hydrochloride was given at a dosage of 10 mg/kg 1 hour before the experiment.

The birds were unanaesthetized and kept in a sitting position during the experiment.

indeed inhibits the *formation* of OA remains to be established.) Consequently, low TA and OA values are found. As might be expected, the action of reserpine is largely counteracted by MAO inhibition. The action of guanethidine may be similar to, though not identical with, that of reserpine.

Preliminary experiments with ^{14}C-dopamine show that this amine also undergoes rapid β-hydroxylation (under formation of noradrenaline) and that this process is no longer detectable after sympathetic postganglionic denervation.

Summary

The rapid formation of octopamine from tyramine by adrenergic nerves is demonstrated *in vivo*. Evidence in support of the existence of both intra- and extra-neuronal binding sites for tyramine is presented. The further investigation of these phenomena may throw light on the pharmacological actions of indirectly acting sympathomimetics

Acknowledgements.

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REFERENCES

Blaschko, H, D Richter & H Schlossmann The oxidation of adrenaline and other amines *Biochem J* 1937, 31, 2187–2196

Burn, J. H & M J Rand The action of sympathomimetic amines in animals treated with reserpine *J Physiol* 1958, 144, 314–336

Carlsson, A., E Rosengren, Å Bertler & J. Nilsson Effect of reserpine on the metabolism of catecholamines *Psychotropic Drugs*, Ed Garattini, S & V Ghetti Amsterdam 1957, pp 363–372

Fleckenstein, A & J H Burn The effect of denervation on the action of sympathomimetic amines on the nictitating membrane *Brit J Pharmacol* 1953, 8, 69–78

Fleckenstein, A & P Stöckle Zum Mechanismus der Wirkungs-Verstärkung und Wirkungs-Abschwächung sympathomimetischer Amine durch Cocain und andere Pharmaka *Arch exper Path u Pharmacol* 1955, 224, 401–415.

Furchgott, R F, S M Kirpekar, M. Rieker & A Schwab Actions and interactions of norepinephrine, tyramine and cocaine on aortic strips of rabbit and left atria of guinea pig and cat *J Pharmacol Exp Ther* 1963, 142, 39–58

Musacchio, J. M. & M Goldstein Biosynthesis of norepinephrine and norsynephrine in the perfused rabbit heart *Biochem Pharmacol* 1963, 12, 1061–1063

Zeller, E A : The role of amine oxidases in the destruction of catecholamines In O. Krayer (Editor) *Symposium on catecholamines* Baltimore 1959, 387–393.

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Material and Methods

The general procedure has been described in earlier papers (SANNER & WORTMAN 1962, SANNER 1963) Campbell's modification of the original Sperber technique was used (CAMPBELL 1960, SPERBER 1949)

A *Animals*

Rhode Island Red chickens 2 years old and weighing 1 9–2 8 kg were used They were kept on commercial chicken food with fresh water ad libitum Generally the birds were fasted over night before an experiment. The birds were pre-treated with 100 mg/kg of an irreversible monoamine oxidase (MAO)-inhibitor (nialamide hydrochloride Niamid ® Pfizer) which was injected into the wing vein about 17 hours before the experiment In one experiment (Expt no 538) 200 mg/kg nialamide were given In another experiment (Expt no 481) Catron ® hydrochloride was given at a dosage of 10 mg/kg 1 hour before the experiment

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Small plastic funnels were sutured over each uretheral orifice under topical anaesthesia (5% Lidocain ointment) The funnels were irrigated with a constant flow of distilled water at a rate of about 0 5 ml/min on each side to prevent their becoming clogged by uric acid The urine was collected from each side separately

B *Drugs and Reagents.*

Dopamine Dopamine (3-hydroxytyramine hydrochloride, Hoffman La Roche, Basle) in powder form was dissolved in 0 9 per cent saline just before injection (pH = 6) The doses given are expressed as the hydrochloride *Tolazoline* Tolazoline (2 benzylimidazoline hydrochloride, Vasodil ®, Leo, Hälsingborg) in powder form was dissolved just before injection in 0 9 per cent saline to give 3 33 mg/ml of the hydrochloride (pH = 6)

Probenecid Probenecid, (p (dipropylsulfamyl)-benzoic acid, Sharp and Dohme, Glenolden, Pa)

I A solution containing 50 mg/ml was made up 2 5 g Probenecid were dissolved in 10 ml of 0 5 N NaOH, the pH was adjusted to 7 4 with 2 ml of 1 0 N HCl and the volume was made up to 50 ml with distilled water

II A solution containing 5 mg/ml was made up 500 mg Probenecid were dissolved in 10 ml of 0 5 N NaOH, the pH was adjusted to 6 3 with 0 1 N HCl, and the volume was made up to 100 ml with 0 9 per cent saline (Expt no 635) All doses of probenecid are expressed as the acid

Phenol red (phenolsulphonphthalein)

I 1 0 mg/ml in 0 9 per cent saline (pH = 7) (For testing birds)

II 1 0 mg/ml in 0 9 per cent saline (pH = 6) (Expt no 567, 569, 572 and 635)

Perchloric acid reagent 100 mg of EDTA (disodium salt) (Merck, Darmstadt) and 200 mg of ascorbic acid (Hoffman–La Roche, Basle) were dissolved in 10 ml of 0 4 N perchloric acid and diluted with 0 4 N perchloric acid to a total volume of 110 ml on the day of the experiment

Nialamide Nialamide, 1 (2 (benzylcarbonyl)ethyl)2-isonicotinylhydrazine hydrochloride (Niamide ®, Pfizer, New York) A solution was prepared just before injection by dissolving the substance in 0 9 per cent saline to make 100 mg/ml of the hydrochloride salt

Catron 1-phenyl-2 hydrazinopropane hydrochloride (Catron ®, Lakeside laboratories, Milwaukee, Wisconsin) in powder form dissolved in 0 9 per cent saline to contain 10 mg/ml of the hydrochloride

Polyethylene glycol (PEG) (Av mol weight 4000) A solution of 125 mg/ml made approximately isotonic by adding 1 65 mg/ml sodium chloride was used

C *Pretesting of Birds*

The birds were generally tested by an injection of phenol red (pH = 7) 0 2 mg/kg into the same leg vein as was later used for dopamine injections A few birds that did not excrete the phenol red with a visible excess on the injected side were discarded

D *Collection of Urine*

... bes con-
exper-
amples
iments the urine was ...
being pooled before analysis

E *Analyses*

Dopamine determination Dopamine was determined by the fluorescence method of CARLSSON & WALDECK (1958) with an Aminco-Bowman spectrophotofluorometer The activation peak was at 325 mμ and the fluorescence peak at 375 mμ. (Uncorrected apparatus values)

The fluorescence was found to be stable for about 24 hours Recovery experiments on diluted urines from each experimental chicken were done with every run except Expt no 635 *The results are shown as "Method Recovery per cent' in the tables* The difference between the amounts excreted by the ipsilateral side and the contralateral side divided by the dose given is taken as the apparent tubular excretion fraction of the injected dopamine (ATEF) (SPERBER 1948)

Phenol red analysis Phenol red excretion was measured in the urine samples after adding an excess of dry Na_2CO_3 A Zeiss Elko photometer was used with filter no S 5351 The excretion of phenol red was calculated in the same way as that of dopamine

Polyethylene glycol analysis PEG was determined by the method of HYDÉN (1955) and HYDÉN & KNUTSON (1959)

Results

1 *Apparent Tubular Excretion Fraction (ATEF) of Dopamine*

In 9 normal chickens pre-treated with a monoamine oxidase inhibitor, dopamine injected at a dose of 50 μg into the leg vein gave a mean ATEF of 19 5 ± 3 8 per cent (table 1)

Since an ATEF above 10 per cent is regarded as indicating tubular secretion, these figures make it probable that dopamine is secreted For all these injections the dopamine was injected in the same volume of 0 9 per cent saline and the injection time was kept the same The recovery of

Table 1

Expt No	Dopamine inj dose μg leg vein	Dopamine excreted μg		ATEF × 100	Recovery percentage of dose	Method Recovery per cent	Inj time min	Volume inj ml	Urine coll time min
		Inj side	Uninj side						
480	50	19 1	1 7	34 7	41 7	98	3	2 5	20
481	50	6 2	0 5	11 5	13 2	105	3	2 5	40
500	50	14 9	2 8	24 2	35 5	87	3	2 5	40
504	50	5 9	1 2	9 4	14 3	91	3	2 5	40
516	50	19 8	7 0	25 7	53 5	70	3	2 5	40
517	50	7 2	0 7	13 1	15 8	69	3	2 5	40
522	50	7 9	5 7	4 3	27 2	100	3	2 5	40
527	50	21 0	2 4	37 0	46 8	71	3	2 5	40
528	50	10 8	2 8	16 0	27 2	80	3	2 5	40
525	150	44 8	6 9	25 2	34 5	101	3	2 5	40
Mean n = 9	50	12 5 ± 2 1	2 8 ± 0 7	19 5 ± 3 8	30 6 ± 4 8	84 1 ± 4 5	3	2 5	–

the injected dopamine was found to be 30 6 ± 4.8 per cent of the dose injected. The recovery of dopamine added to diluted chicken urine (method recovery) was 84.1 ± 4 5 per cent. The table also shows that neither dose nor collection time are critical. *Urine flows were not significantly altered by the dopamine injections.*

2. *Dopamine – Tolazoline Injections.*

In another series of experiments dopamine was first injected into one leg vein and the urinary excretion of dopamine over 20 or 40 minutes was then determined. Ten minutes after the end of the first urine collection period the same dose of dopamine together with 5000 μg of tolazoline was injected into the same leg vein. The injection time and the volume injected were kept equal to those for the previous dopamine injection in each bird, and the urinary excretion of dopamine was determined over the same length of time for both dopamine injections. At this dose of tolazoline the excretion of dopamine was much less than that in previous

Table 2.

Expt No	Dose inj, μg, leg v		ATEF × 100 Dopamine	Recovery percentage of Dopamine dose	Method recovery per cent	Inj time min	Volume inj ml	Urine collect time *min*
	Dopamine	Tolazoline						
530	100	–	21 0	26 3	94	3	2 5	20
	100	5000	4 7	10 7		3	2 5	20
533	100	–	4 5	18 0	86	3	2 5	20
	100	5000	3 0	13 5		3	2 5	20
535	100	–	3 9	13 1	96	3	2 5	20
	100	5000	2 1	7 0		3	2 5	20
537	100	–	18 9	24 7	76	3	2 5	20
	100	5000	4 5	13 6		3	2 5	20
538	100	–	16 8	23 1	94	3	2 5	40
	100	5000	4 8	14 5		3	2 5	40
542	100	–	20 0	34 6	54	3	2 5	40
	100	5000	−2 3	9 5		3	2 5	40
543	100	–	39 4	45 5	86	3	2 5	40
	100	5000	10 5	28 3		3	2 5	40
544	100	–	42 2	42 2	85	3	2 5	40
	100	5000	23 2	24 3		3	2 5	40
mean n = 8	100	–	20 8±1 4	28 4±4 0	83 9±4 8	3	2 5	–
	100	5000	6 3±2 7	15 2±2 6		3	2 5	

control tests on the same experimental animal In the 8 birds used the dose of dopamine was kept at 100 µg In these 8 experiments, the mean ATEF of dopamine was 20 8 ± 1 4 per cent when dopamine alone was given and 6 3 ± 2 7 per cent when dopamine and tolazoline were given together All birds were pre treated with nialamide The recovery of the injected dopamine was found to be 28 4 ± 4 0% of the dose for dopamine alone and 15 2 ± 2 6% when dopamine and tolazoline were given together (table 2) Urine flows were not significantly altered by the injections

3 *Probenecid Experiments*

In these experiments probenecid was injected in different ways to test whether probenecid can inhibit the excretion of dopamine In one of them (Expt no 545) dopamine was first injected into one leg vein and the urine was collected for determining the urinary excretion of dopamine Then a single dose of 50 mg/kg probenecid was injected into the wing vein, and 1 hour later the same dose of dopamine was injected into the same leg vein as before The urine was collected for an equal time after both the injections, and the dopamine excretion was determined In one further experiment (Expt no 549) dopamine was first injected into one leg vein and dopamine excretion was determined Probenecid was then infused (5 mg/min) into the wing vein throughout the following injection procedure Probenecid was infused for a period of 10 minutes before the second injection of dopamine into the leg vein was made The probenecid infusion was then continued for the rest of the experiment (40 minutes) No evidence was found for any interference by probenecid upon dopamine excretion (table 3)

In another series of experiments (Expt no 567, 569, 572) dopamine was given into the leg vein together with phenol red Two injections were

Table 3

Expt No	Dose injected: Dopamine leg vein µg	Dose injected: Probenecid wing vein	ATEF × 100 Dopamine	Recovery percentage of dose	Method recovery per cent	Inj time min	Volume inj ml	Urine collect time min
545	100		23 1	27 4	78	3	2 5	40
	100	50 mg kg 1 hr	42 3	44 6		3	2 5	40
549	100	–	48 1	48 5	103	3	2.5	40
	100	5 mg min (50 min)	34 5	42 7		3	2 5	40

Table 4.

Expt No	Dose injected, *kg leg v*		Probenecid wing vein (pH = 7.4)	*ATEF × 100*		Recovery percentage of dose		Method Recovery per cent	Inj time min	Volume inj ml	Urine Collection time min
	Dopamine	Phenol Red (pH – 6)		Dopamine	Phenol Red	Dopamine	Phenol Red				
567	100	800	–	40.6	43.4	41.5	70.5	99	3	3.3	40
	100	800	50 mg/kg –20 min	49.6	4.2	51.3	21.9		3	3.3	40
569	100	800	–	11.5	21.5	16.4	64.4	118	3	3.3	40
	100	800	50 mg/kg –20 min	28.6	2,3	30.7	19.6		3	3.3	40
572	100	800	–	54.3	31.2	60.4	62.0	92	3	3.3	40
	100	800	50 mg/kg –20 min	31.5	–5.8	41.6	26.9		3	3.3	40
mean n – 3	100	800	–	35.5	32.0	39.4	65.6	103	3	3.3	40
	100	800	50 mg/kg –20 min	36.6	0.2	41.2	22.8		3	3.3	40

Table 5

Expt No	Infusion rate leg vein			ATEF × 100		Recovery percentage of dose		Infusion time min	Volume infused ml	Urine collection time min
	Dopamine μg/min	Phenol Red μg/min (pH – 6)	Probenecid mg/min (pH = 6 3)	Dopamine	Phenol red	Dopamine	Phenol Red			
635	2 5	20	–	10 2	15 9	11 6	30 5	40	10	40
	2 5	20	1 025	26 4	7 1	27 0	23 5	40	10	40

Table 6

Number of expts	Time minutes	Urine flow μl/min		PEG excretion, mg/min		PEG excretion ratio inj side to Uninj side
		Inj side	Uninj side	Inj side	Uninj side	
6 mean ± S E M	(−30) (−20)	62 0 ± 11 5(5)	58 6 ± 7 7(5)	4 85 ± 1 05	4 77 ± 1 09	1 05 ± 0 12
	(−20) (−10)	76 0 ± 15 2	81 8 ± 17 6	8 57 ± 0 94	8 38 ± 0 79	1 02 ± 0 06
	(−10) 0	79 3 ± 29 1	87 2 ± 15 8	8 57 ± 0 74	8 40 ± 0 70	1 03 ± 0 05
100 μg Dopamine 0–2,5 min leg vein	0– 5	80 5 ± 11 0	84 8 ± 14 1	9 48 ± 0 69	8 75 ± 0 92	1 11 ± 0 07
	5–10	88 8 ± 19 8	96 2 ± 18 6	9 20 ± 0 47	9 72 ± 0 66	0 96 ± 0 06
	10–20	73 2 ± 9 2	76 8 ± 12 9	9 28 ± 0 76	8 73 ± 0 89	1 09 ± 0 07
	20–30	74 5 ± 4 9	79 2 ± 9 6	9 27 ± 0 42	9 75 ± 0 68	0 97 ± 0 08
	30–40	76 2 ± 9 3	82 0 ± 11 1	10 57 ± 0 62	10 32 ± 0 64	1 04 ± 0 05

given to each bird First one injection of dopamine phenol red was given without any inhibitor present, then one injection of dopamine-phenol red was given to a chicken pre injected with 50 mg/kg probenecid into the wing vein 20 minutes before Probenecid pre treatment produced no change in the excess excretion of dopamine, but the tubular secretion of phenol red was completely inhibited (table 4)

Finally, one experiment (Expt no 635) was performed in which probenecid (pH = 6,3) was unilaterally injected together with dopamine and phenol red into one leg vein Here also no inhibition by probenecid of dopamine excretion was found (table 5)

4 *Polyethylene Glycol Infusions*

In order to test the influence of dopamine on the glomerular filtration rate the experiments described below were done In a series of 6 normal chickens not pre treated with any MAO inhibitor, isotonic polyethylene glycol (PEG) at a constant rate of 31 mg/min (0 25 ml/min) was infused into one wing vein throughout the experiment A control period of 30 minutes was allowed before 100 µg of dopamine were given into one leg vein at an injection rate of 40 µg/min The urine was collected for another 40 minutes for PEG analyses as well as for urine volume measurements The results are given in table 6 The PEG excretion ratios (injected side/uninj side) were variable but were not far from 1

Discussion

Dopamine has been shown to be excreted in excess on the injected side In order to test whether or not dopamine is actively secreted by the common base transport system, tolazoline was used as an inhibitor and was injected into the leg vein together with dopamine With tolazoline present the ATEF of dopamine decreased, probably by competitive inhibition of the transport system, to values less than 10 per cent Tolazoline at the dose given has previously been shown to inhibit serotonin secretion but not phenol red secretion

In the probenecid experiments carried out here, phenol red was included as a test of probenecid activity Probenecid did not show any inhibition of dopamine excretion in these experiments, but strongly inhibited the phenol red excretion as expected These experiments are
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experiments showed that DL-adrenaline or its metabolites were excreted by the renal tubules and also the somewhat unexpected finding that this excretion was not affected by an inhibitor of the base transport system Instead, it was inhibited by probenecid, but not by another inhibitor of organic acid transport, bromcresol green The probenecid was infused into the leg vein of the chicken together with DL-adrenaline and p-aminohippurate (PAH) As can be seen from the dopamine experiments described here, probenecid was tested by a few different injection techniques, among them one similar to that used by RENNICK (1962) (Expt no 635) Our experiments did not show any inhibition of dopamine excretion by probenecid

A probable explanation of Rennick's findings is that she did not use an inhibitor of monoamine oxidase and was in fact studying the excretion of catecholic acids *)

Dopamine can also act by interference with the blood vessels of the kidneys, since it is a vasoactive substance It could possibly interfere with the arterioles imbedded in the parenchyma and cause a local increase in glomerular filtration rate on the side injected with dopamine This might simulate tubular excretion

This was also tested The results did not show any significant influence of dopamine on the glomerular filtration rate (table 6)

The most probable excretion mechanism of dopamine is tubular secretion involving the common base transport system

Summary

Dopamine at a dose of 50–150 µg was injected into the leg vein of chickens All birds were pre treated with a MAO inhibitor (nialamide, Niamide ®) except one bird for which the substance JB 516 (Catron ®) was used Dopamine was found to be excreted unilaterally in excess on the dopamine injected side The excretion of dopamine was inhibited by the simultaneous administration of tolazoline at a dose of 5 mg into the leg vein Probenecid at a dose of 50 mg/kg given into the wing vein 20 minutes before leg vein injection of dopamine and phenol red produced strong inhibition of phenol red excretion, but left dopamine transport unchanged Dopamine appears to be actively secreted by the common base transport system

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REFERENCES

Campbell D E S Improved Method for Collecting and Measuring Ureteral Urine Flow in the Chicken *Acta pharmacol et toxicol* 1960 17 205 212

Carlsson A & B Waldeck A Fluorimetric Method for the Determination of Dopamine (3 Hydroxytyramine) *Acta physiol Scand* 1958 44 293 298

Euler U S v & S Hellner Excretion of Noradrenaline Adrenaline and Hydroxytyramine in Urine *Acta phys ol Scand* 1951 22 161 167

Sweden 1955 22 139 145

Hydén S & P G Knutson Renal Clearance and Distribution Volume of Polyethylene Glycol and Inulin in the Chicken *Ann Roy Agricult Coll Sweden* 1959 25 253 259

Nechay B R & E Sanner Interference of Reserpine with the D uretic

ives and Phenol *l Sweden* 1948

15 317 349

Sperber I Investigations on the Circulatory System of the Avian Kidney *Zool B drag Uppsala* 1949 27 429–448

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